Sleep and fatigue during chronic viral infection

by

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DEDICATION

This work as a whole is dedicated to those who suffer with post-viral chronic fatigue, in hopes that one day we will have a cure.

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ABSTRACT

INTRODUCTION: Human gammaherpesvirus (GHV) infections, such as with Epstein-Barr Virus (EBV), are associated with fatigue and elevated concentrations of cytokines. Murine GHV-68 (MuGHV) has been used as a model of EBV. However, the behavioral impact of infection with MuGHV is unknown.

METHODS I: Male C57BL/6J mice (25 – 28 g at time of surgery) were housed in cages containing running wheels and infrared detectors to measure activity. Mice were administered vehicle intranasally (I.N.) at dark onset and recordings of EEG, body temperature and activity were made for 4 days. Mice were inoculated I.N. at dark onset with 400 PFU (n=13) or 40,000 PFU (n=8) of MuGHV and recordings continued for 30 days. Mice were then injected intraperitoneally (I.P.) at dark onset with vehicle and 24 h control recordings obtained. The mice were injected I.P. with lipopolysaccharide (LPS) and recordings continued for 5 days.

METHODS II. Mice housed in the same conditions were inoculated I.N. with vehicle and sacrificed after 4 days or inoculated with 400 PFU or with 40,000 PFU MuGHV and sacrificed 5, 9, or 26 days after inoculation. Brain tissue, blood, spleen, and lung were collected, and stored at -80 °C until assay.

RESULTS I. Infected mice showed decreases in wheel running activity, core body temperature, food intake and body weight. Mice inoculated with 40,000 PFU of MuGHV

had increases in NREM sleep, sleep fragmentation, and wakefulness during the peak of viral replication. Responses of infected mice to LPS were exacerbated.

RESULTS II: Cytokine concentrations and profiles in brain and the periphery were altered during infection. The alterations in cytokine profiles differed in a complex manner depending on dose of MuGHV.

CONCLUSIONS: Based upon reductions in wheel running, these results indicate that mice become chronically fatigued during infection with MuGHV. Chronic infection is associated with long-term changes in thermoregulatory mechanisms and the ability of the immune system to respond normally to subsequent challenge. Virus-induced alterations in central cytokine profiles with respect to those in the periphery suggest potential mechanisms that may lead to fatigue. These studies demonstrate the utility of MuGHV as a model for post-viral fatigue infection.

CHAPTER I:

INTRODUCTION

Chronic fatigue and Epstein-Barr Virus

Chronic fatigue is a widespread, disabling symptom that decreases quality of life and productivity in the workplace (Bombardier & Buchwald, 1996). While the etiology of chronic fatigue syndrome (CFS) remains elusive, it is often hypothesized that many patients develop symptoms of CFS after an infection with a gammaherpesvirus (GHV) such as Epstein-Barr virus (EBV). Chronic fatigue can be triggered by many factors, including viral or bacterial infection, surgery, or depression (Afari & Buchwald, 2003). If fatigue is not attributed to a primary illness, is severe and unremitting for a period of six months or more, and is not substantially alleviated by rest, it is classified as chronic fatigue syndrome (CFS) (Fukuda et al., 1994). In CFS, chronic fatigue can be accompanied by symptoms such as sore throat, lymph node pain, muscle pain, joint pain, headache, and memory difficulties (Afari et al., 2003; Fukuda et al., 1994). Most CFS patients complain of disrupted sleep, including difficulty maintaining sleep, a need for daytime naps, and feeling unrefreshed upon awakening, as well as mental health issues such as anxiety and depression (Buchwald, Pascualy, Bombardier, & Kith, 1994; Moldofsky, 1989). It is estimated that CFS affects approximately 800,000 Americans of all socioeconomic classes and also affects relatively equal amounts of men and women.

The annual economic impact of CFS is estimated at $9.1 billion due to loss of productivity (Reynolds, Vernon, Bouchery, & Reeves, 2004), however its impact due to depression as a result of chronic fatigue is still unknown. In addition, individuals with chronic fatigue and CFS are reported to make an average of 21 primary care visits per person each year, which poses a large burden on the health care system (Bombardier et al., 1996). CFS leads to a higher degree of impairment than other chronic illnesses (Bombardier et al., 1996) and there are very few effective treatments available (Afari et al., 2003). Chronic fatigue and CFS are difficult to study in humans due to the heterogeneous patient population and the variety of triggering events (Afari et al., 2003; Evans, 1991; Krupp, Mendelson, & Friedman, 1991; Kyle & DeShazo, 1992).

EBV is a ubiquitous human GHV that establishes life-long latency. The hypothesis that EBV is involved in CFS derives, in part, from observations that individuals with CFS have higher antibody titers to EBV (Glaser et al., 2005; Matthews, Lane, & Manu, 1991; Swanink et al., 1995). Though some studies do not demonstrate a relationship between EBV and CFS (Buchwald, Pearlman, Umali, Schmaling, & Katon, 1996; Koelle et al., 2002; Mawle et al., 1995), the numbers of CFS patients with high EBV titers may be under-represented due to poor test specificity, variation in viral gene expression, and low viral gene expression during the latent period (Glaser et al., 2005). Data indicate that 95% of all adults have antibodies against EBV (Rickinson & Kieff, 2001; Mandell, Bennett, & Dolin, 1995). Transmission of EBV can occur through blood products, transplantation, saliva, and sexual activity (Mandell et al., 1995), which makes prevention of the spread of this virus extremely challenging. Vaccination efforts have not been successful due to EBV's complex stages and protein expression, mainly the

variability in latency phenotypes of EBV (Khanna, Moss, & Burrows, 1999; Khanna, Burrows, & Moss, 1995; Cohen, 2000), and medical interventions are rather ineffective in decreasing symptom severity (Gershburg & Pagano, 2005). Thus a large proportion of the general population is at risk for post-viral fatigue in response to EBV, and also at risk for developing depression as a result of fatigue. Many studies suggest EBV may be central to the pathogenesis of CFS and may perpetuate the chronic state of fatigue (Glaser & Kiecolt-Glaser, 1998; Jones, 1988; Natelson et al., 1994).

Pro-inflammatory cytokines, such as IL-1β, TNFα, IFN-γ, and IL-6 and are principle mediators of the effects of GHV infection (Sarawar, Lee, & Giannoni, 2004). These cytokines also induce sickness behavior, as manifest by fatigue, fever, malaise, and arthralgia (Dantzer, 2001; Dantzer et al., 1998; Dantzer et al., 1999a; Vollmer-Conna et al., 2004). In addition to their role in sickness behavior after an immune challenge, these pro-inflammatory cytokines regulate and/or modulate normal sleep (Opp, 2005). Patients with CFS have higher IL-1 and IL-6 levels that coincide with periods of fatigue (Cannon et al., 1999). This relationship among IL-1, IL-6 and fatigue does not appear in either normal subjects or CFS patients during exercise-induced fatigue (Gupta, Aggarwal, & Starr, 1999). In addition, patients with CFS report poor quality of sleep, including insomnia, awakenings during the night, and unrefreshing sleep (Unger et al., 2004; Fukuda et al., 1994). It is possible that elevated circulating cytokines during EBV infection result in CFS and the post-viral symptoms of fatigue and daytime sleepiness. The following studies will address this concept.

It is difficult to study EBV and post-viral chronic fatigue in humans due to the heterogeneity of the patient population (age, stage of infection, variable gene expression

during latency, unpredictability of fatigue trigger, and the time of infection), and subject sampling methods (Glaser et al., 2005). In addition, fatigue can be subjective and is assessed through self-report (Hossain et al., 2005). Fatigue is also difficult to assess in animals (Hossain et al., 2005). Previously published animal models of chronic fatigue have used bacterial or viral infections, such as *Brucella abortus*, *Cryptosporidium parvum* or influenza virus (Sheng, Hu, Lamkin, Peterson, & Chao, 1996; Ottenweller et al., 1998), as the infectious agent, or have used non-infectious challenges such as 2'-deoxyuridine 5'triphosphosphatase (dUTPase, an enzyme encoded by EBV) (Glaser et al., 2005; Fleischmann, Kremmer, Greenspan, Grasser, & Niedobitek, 2002). *Brucella* or influenza virus do not produce latent infections, and repeated administration of dUTPase does not constitute a true infection. As such, these challenges are unable to model many of the sequelae that are concomitant with chronic viral infection. Furthermore, these previously reported studies have primarily focused on immune responses, and not the relationships between immune responses and behavior. These projects focused on cytokines as mediators of fatigue in response to viral infection. The following experiments assessed sleep-wake behavior, fatigue, and cytokine profiles of mice infected with MuGHV during both active and latent stages of viral infection. These studies also determined the impact of a secondary immune challenge on mice in the latent phase of infection with this virus.

Relevance to Mental Health

Fatigue is a hallmark symptom of depression and is reported in about 66% of patients diagnosed with depression (Johnson, 2005). In population studies, depression

generally predicts fatigue, and conversely fatigue predicts onset of depression (Johnson, 2005). In addition, anxiety and depression are frequently comorbid symptoms in subjects suffering from chronic fatigue syndrome (Kirmayer, Robbins, Dworkind, & Yaffe, 1993). Cytokines are implicated as mediators of fatigue as well as the onset and maintenance of depression (Dantzer, Wollman, Vitkovic, & Yirmiya, 1999b). Cytokines induce behavioral changes that are consistent with depression in humans (Anisman & Merali, 1999,Dantzer et al., 1999b) high levels of circulating cytokines are found in patients with depression, and administration of cytokines into healthy volunteers induces depressive-like symptoms and fatigue (Maier & Watkins, 2000). Similarly, healthy volunteers report anxiety and depressive-like feelings after endotoxin administration, and the magnitude and extent of these feelings directly correlate with the secretion of TNF-α and IL-6 (Reichenberg et al., 2001). Finally, cytokines can induce fatigue, and fatigue itself may lead to depression (Johnson, 2005). However, the mechanisms by which cytokines, fatigue, and depression interact remain largely unknown. By elucidating potential mechanistic contributions of cytokines to post-viral fatigue using an animal model, these studies have furthered the understanding of the relationships among cytokines, fatigue, and multiple facets of mental health.

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CHAPTER II:

MURINE GAMMAHERPESVIRUS 68: A MODEL FOR THE STUDY OF EPSTEIN-BARR VIRUS INFECTIONS AND RELATED DISEASES.[1]

Abstract

Gammaherpesviruses (GHVs) are double-stranded DNA viruses that cause acute disease and then persist in the body in a latent form (Ackermann, 2006). Like other herpesviruses, GHVs (Table 1) are classified based on their genome organization (Ackermann, 2006) and are composed of a large double-stranded linear DNA genome encased in a protein capsid that is in turn wrapped in a lipid bilayer membrane envelope (Damania, Choi, & Jung, 2000). Epstein-Barr virus (EBV) is a ubiquitous human GHV that causes both acute and chronic disease (Cohen, 2000). The molecular biology of EBV has been characterized extensively by use of in vitro systems (Callan, 2004; D'Addario, Ahmad, Morgan, & Menezes, 2000; Haan, et al., 2001). The strong species specificity of GHVs has precluded using human GHVs in animal models, making the study of their pathogenesis difficult. However, in 1980, a murine GHV (MuGHV, also known as MHV68 and γHV68) was identified as a natural pathogen of bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus flavicollis*) that was able to infect laboratory mice

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[1] This chapter was published in its entirety. The complete citation is:

{BLASKOVIC1980}. Experimental MuGHV infections in laboratory mice share many features of EBV infections in humans, including facets of the clinical human syndrome known as infectious mononucleosis (Flano, Woodland, & Blackman, 2002). These features make MuGHV a valuable experimental model for studying the pathophysiology of a GHV in a natural host.

Epstein-Barr virus

EBV (also known as human herpesvirus 4) is a ubiquitous human virus that infects B cells in humans and New World nonhuman primates (Ackermann, 2006). In humans, EBV causes acute disease and establishes life-long latency (Rickinson & Kieff, 2001). EBV infections are associated with the syndrome known as infectious mononucleosis and, less frequently, with the development of B-cell lymphoproliferative disorders, several malignancies—including Burkitt lymphoma, Hodgkin disease, and nasopharyngeal carcinoma—and lymphomatoid granulomatosis, a form of chronic lung disease (Table 2) (Iwatsuki et al., 2004). About 95% of the United States population has been exposed to and carries antibodies against EBV (Rickinson et al., 2001). Despite humoral and cellular immune responses by the host, EBV nonetheless establishes latent infection (Stevenson & Efstathiou, 2005).

Routes of transmission for EBV include blood products, organ transplantation, saliva, and sexual activity (Hess, 2004). In the susceptible host, EBV establishes an initial lytic infection in the lung epithelium that resolves within days. This acute phase is followed by the establishment of a lifelong latent state, predominantly in the B cell compartment in the spleen, with latency maintained under the control of the host immune

system. Reactivation allows infectious virus particles to spread from B cells to the oropharyngeal mucosa, where virus can be shed and transmitted to new hosts (Stevenson et al., 2005; Damania et al., 2000).

During the lytic phase of infection, EBV uses several mechanisms to evade host defense processes. EBV enters neutrophils, penetrating the nucleus and triggering apoptosis (Larochelle, Flamand, Gourde, Beauchamp, & Gosselein, 1998). In addition, EBV infection reduces the phagocytic activity of monocytes, interferes with the function of dendritic cells, and inhibits differentiation of monocytes into mature dendritic cells (Levitsky & Masucci, 2002). EBV infection also triggers the secretion of interleukin (IL) 8 and macrophage inflammatory protein 1α (MIP1α), which attract both B and T cells to the site of inflammation (McColl, Roberge, Larochelle, & Gosselin, 1997), thereby increasing the pool of cellular targets for viral infection.

EBV modulates T lymphocyte responses by suppressing the production of antigen receptors on effector T cells (Chen et al., 2001). In this manner, EBV establishes an immunoprivileged site for persistence in secondary lymphoid tissue and respiratory epithelium (Chen et al., 2001). In latent EBV infection, viral gene expression is restricted and reduced (Levitsky et al., 2002). An important factor in this process is transformation of the linear viral genome into the circular form known as an episome. The episomal configuration limits translation and expression of viral proteins, thereby limiting the presentation of viral epitopes to T cells, curtailing expression of signals that would trigger a host response, and allowing the virus to remain undetected in cells (Callan, 2004; (Flano, Woodland, & Blackman, 2002); Flano, Kim, Moore, Woodland, & Blackman, 2003). If host immune cells do recognize the virus, the virus can, in turn, inhibit antigen

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processing via a Gly–Ala repeat in EBV nuclear antigen 1 that alters the kinetics of antigen processing and prevents apoptosis of latently infected cells (Lagunoff & Carroll, 2003; Levitskaya, Sharipo, Leonchiks, Ciechanover, & Masucci, 1997).

Although the molecular biology of EBV has been characterized extensively, infection-induced disease and its pathogenesis are difficult to study in EBV-infected human populations due to the chronic and latent properties of the virus, human variation in environment and genetics, the well-documented impact of stressors on viral recrudescence, ethical concerns, and the complexity of the symptoms. For example, EBV infection in humans often is associated with fatigue and excessive sleepiness (Ablashi, 1994; Guilleminault, 1989; Josephs et al., 2000; Schumann et al., 1990; White et al., 2001). Such symptoms could be related to immune stimulation or dysfunction, neural-endocrine homeostatic imbalance, or both, as produced secondary to acute and chronic viral infection (Cleare, 2003; Papanicolaou et al., 2004). However, the pathogenesis, progression, and therapy of complex and debilitating symptoms like fatigue and nonrestorative sleep are difficult to study in human populations with EBV. Therefore, using a mouse model to study the pathogenesis of fatigue and sleepiness in relation to latent viral infection and immune dysfunction would be highly beneficial for understanding the mechanisms that link GHVs and fatigue. GHVs are highly species-specific; thus EBV does not infect laboratory mice (Haan, Aiyar, & Longnecker, 2001). Nonhuman primates can be infected with a rhesus lymphocryptovirus, a related gammaherpesvirus, but marked differences in the progression of the infection complicate its use as a model for EBV (Moghaddam et al., 1997). A valid animal model of GHV infection would facilitate the study of associated pathogenesis and disease states by

allowing control and evaluation of host factors (such as genetics and environment) and the assessment of pathogenic features as the disease progresses. In the following sections, we discuss similarities of EBV and MuGHV that make MuGHV-infected mice useful for studying the behavioral consequences of GHV infection in relation to immunologic mechanisms.

Murine gammaherpesvirus

MuGHV was identified in 1980 (Blaskovic, Stancekova, Svobodova, & Mistrikova, 1980) in 2 species of rodents (*A. flavicollis* and *C. glareolus*) and was shown to readily infect several strains of laboratory mice (Blaskovic et al., 1980; Rajcani et al., 1985). The virus was classified as a GHV in light of the sequence and organization of the viral genome and its virion architecture (Table 1) (Virgin et al., 1997). MuGHV shares important structural and biologic features with human GHVs, including EBV (Nash & Sunil-Chandra, 1994; Virgin et al., 1997). MuGHV and EBV have common blocks of conserved genes and are similar in terms of establishment and clearance of the acute infection, establishment of latency, and the immunologic responses they elicit in the host. In addition, MuGHV and EBV both show epithelial and B-cell tropism, virus-driven B-cell activation and proliferation, and a syndrome of acute infectious mononucleosis (Flano et al., 2002; Nash et al., 1994). Because an in vivo system is essential to studying the complex interplay between infection, immunology, and disease symptoms, including disease-related behavioral changes, MuGHV is a valuable experimental model for studying the pathophysiology and related behavioral outcomes of GHV infections in vivo.

Genetics and immune evasion strategies of MuGHV. The MuGHV viral genome is a linear double-stranded DNA molecule consisting of 118,237 basepairs of virally unique sequences flanked by multiple copies of 1213-basepair terminal repeats that encode for at least 100 viral proteins (Virgin et al., 1997). Within the genome, 80 of these genes are largely colinear with the EBV genome (Virgin et al., 1997). The genome is encased in a nucleocapsid and encodes several membrane proteins that allow the virus to enter the host cell, avoid immune targeting, and move from one cell to another (Bortz et al., 2003). In addition, the MuGHV genome encodes virus-specific open reading frames that are shared by EBV, including genes that are considered important for viral tropism, latency, and transformation (Virgin et al., 1997). These proteins regulate the expression of viral genes, facilitate replication of viral DNA, and influence host immune responses (Virgin et al., 1997). The receptors used by MuGHV remain unknown.

Although the natural route of MuGHV infection has not yet been defined, the respiratory route is presumed to be the main method of transmission for MuGHV. However, virus transmission between infected and uninfected laboratory mice housed together has not been reported, to our knowledge. Whether transmission in research settings failed due to differences between laboratory and wild rodents, attenuation of laboratory stocks of virus, or other factors is unknown. Adult laboratory mice inoculated intranasally with MuGHV develop an initial infection in alveolar epithelial and pulmonary mononuclear cells, both of which also are involved in EBV infection (Ackermann, 2006). Other studies have used intraperitoneal injection of virus; this method generates similar splenic peak viral loads as does intranasal inoculation across a wide range of doses (Tibbetts, McClellan, Gangappa, Speck, & Virgin, 2003b). Viral

dosages range from 0.1 to 10^6 plaque forming units (PFU) without appreciable mortality, and route of infection and viral dose do not appear to affect latent viral titers in the spleen (Tibbetts et al., 2003b). The efficacy of wide dose ranges and various routes of administration facilitate the use of MuGHV as a model for studying the effects of a GHV on host immune responses and associated behavioral changes, such as the fatigue and altered sleep that accompany human EBV infections.

The acute phase of MuGHV infection includes production of infectious virus in and lysis of alveolar epithelial cells. Viral titers in the lung peak between days 5 and 10 after infection, and viral clearance in the lung occurs within 9 to 15 d after infection (Cardin, Brooks, Sarawar, & Doherty, 1996; Tibbetts et al., 2003a) (Figure 1). The acute (lytic) phase of infection generates a host inflammatory response in the lung that persists for as long as 30 d after infection. Monocytosis and macrophage activation peak at 3 d after infection, eliciting the production of IL12, the resultant production of interferon (IFN) γ, and subsequent T cell activation (Elsawa & Bost, 2004) These processes promote pulmonary viral clearance, leukocytosis, and splenomegaly (Dutia et al., 2004; Elsawa et al., 2004; Sunil-Chandra, Efstathiou, Arno, & Nash, 1992). CD8+ T cells with Vβ4+ markers dominate the early stages of viral infection, although the degree of expansion varies with the haplotype of the major histocompatibility complex (Pivik et al., 1993; Hardy et al., 2001). Analysis of recombinant inbred mice revealed 2 quantitative trait loci that are associated with the magnitude of Vβ4+CD8+ T cell expansion, which is involved in lymphocytosis (Hardy et al., 2001).

Like EBV, MuGHV uses various mechanisms to curtail the host defenses, allowing for lifelong infection. For example, the M11 protein inhibits apoptosis, and

RCA inhibits complement activation (Wang & Barks, 1999)(Kapadia, Molina, van, V, Speck, & Virgin, 1999). MuGHV potentially inhibits inflammatory chemokine responses via a chemokine-binding protein, M3, which can delay or decrease the host's response to the virus (van, V et al., 2002). The M3 protein also causes the failure of localization of memory CD8+ T cells to sites of virus reactivation (Obar et al., 2004). In addition, MuGHV induces the release of endogenous IL10, which limits the leukocytosis of B cells infected with the virus (Peacock & Bost, 2001). Therefore like EBV, MuGHV uses immune evasion strategies that modulate inflammatory responses and facilitate viral replication.

Latent infection and chronic disease. Like other herpesviruses, MuGHV can maintain long-term latency in its host. Although the pulmonary inflammation generated in response to lytic infection resolves during the second week, coincident with viral clearance from the lung (Elsawa et al., 2004), antigen persists in the lungs for as long as 30 d after infection, suggesting long-term persistence of the virus (Sunil-Chandra, Efstathiou, Arno, & Nash, 1992). Spleen, lymph nodes, and bone marrow also harbor latent virus, primarily in B lymphocytes, macrophages, and dendritic cells (Flano, Husain, Sample, Woodland, & Blackman, 2000; Flano et al., 2003; Flano et al., 2002; Sunil-Chandra et al., 1992). In the spleen, latent virus is maintained in all 3 of these cell types for at least 3 mo after infection, with the highest frequencies in memory and germinal center B cells (Flano et al., 2005). Relatively constant numbers of latently infected B cells are present in the spleen at different times over the lifespan of MuGHV-infected mice (Tripp et al., 1997).

After intranasal infection with MuGHV, pulmonary titers of lytic virus detected

by plaque assay peak between days 6 to 9, and lytic virus is cleared by days 10 to 14 (Flano et al., 2003). However, between 10,000 and 100,000 cells in the lung carry the viral DNA by day 3 after infection (Flano et al., 2003). This frequency is essentially unchanged for at least 21 d (Flano et al., 2003). An analysis of sorted populations of lung cells (B cells, macrophages, dendritic cells, and 'null' cells) revealed that all subsets contain latent virus (Flano et al., 2003).

MuGHV latency usually is studied in the spleen (Flano et al., 2000; Flano et al., 2003; Sunil-Chandra et al., 1992). Latency typically is assessed by measuring the ability of cell-associated virus to form lytic plaques in vitro (Flano et al., 2000). Latency also can be estimated by measuring the frequency of cells harboring viral genome through use of a limiting-dilution polymerase chain reaction assay. In the absence of lytic virus in the host, this assay measures total (that is, 'genome-positive') latency (Flano et al., 2003; Flano et al., 2002; Weck, Kim, Virgin, & Speck, 1999). Based on plaque formation, limiting-dilution assays can provide a quantification of both preformed infectious virus particles as well as cells that reactivate latent MuGHV within the same tissue sample, allowing the investigator to distinguish between lytic and latent virus. In contrast, polymerase chain reaction-based assays quantify overall amounts of virus in a sample without distinguishing between lytic and latent virus (Virgin, Presti, Li, Liu, & Speck, 1999). At the beginning of the latent phase, virus is present in 1 in 10^3 spleen cells at day 14 after infection, but this titer then declines rapidly, such that viral load at 4 wk after infection is barely above the assay limit of detection (about 1 in 10^6 to 1 in 10^7 spleen cells) of a limiting-dilution polymerase chain reaction assay (Cardin et al., 1996). The development of latency is associated with splenomegaly, polyclonal B cell activation,

autoantibody production, and lymphocytosis. The lymphocytosis consists largely of CD8+ T cells, a large proportion of which express Vβ4+ T cell receptor (Sunil-Chandra et al., 1992).

The virus is able to maintain its presence in the spleen during latency through an open reading frame within the viral genome (ORF 73) that encodes latency-associated nuclear antigen. This protein binds to the viral-latency associated origin of replication and the host cell chromosome, thereby promoting maintenance of the viral episome during latency by partitioning the viral genome to daughter cell DNA during mitosis (Moorman, Willer, & Speck, 2003). Because viral gene expression is highly constrained during latency, the virus and its low levels of expressed proteins present a difficult target for immune intervention (Nash et al., 1994).

Viral reactivation. Host immune control normally prevents reactivation of latent virus. Both humoral and cellular immune processes contribute to the maintenance of latency (Gangappa, Kapadia, Speck, & Virgin, 2002; Kim, Flano, Woodland, & Blackman, 2002; Stevenson, 2004; Stewart, Usherwood, Ross, Dyson, & Nash, 1998). CD28 is a costimulatory molecule that inhibits humoral immunity and the coordination of T and B cell responses (Kim et al., 2002). CD28$^{-/-}$ mice do not form antibodies but nonetheless control lytic MuGHV replication; latency is established in CD28$^{-/-}$ mice, but depletion of T cells in CD28$^{-/-}$ mice with latent infection allows viral reactivation, as indicated by the rapid appearance of lytic virus in both lung and spleen (Kim et al., 2002). In contrast, T cell-depletion of infected C57BL/6 mice, which have high levels of neutralizing antibodies, does not allow viral reactivation (Kim et al., 2002). Reactivation of both EBV and MuGHV occurs via the initiation of viral replication in latently infected

B cells (Cohen, 2000).

The processes that trigger reactivation of latent virus are largely unknown. Signaling through nuclear factor κB is postulated to inhibit reactivation; absence of this signaling releases inhibition of replication and allows viral reactivation (Brown et al., 2003). Ex vivo exposure of latently infected cells to lipopolysaccharide has been shown to induce reactivation (Moser, Upton, Gray, & Speck, 2005) Latent virus undergoes apparently random reactivation in some host cells, inducing the production of low levels of cytokines (Moser et al., 2005). This process occurs in macrophages, B cells, and dendritic cells after short-duration latency (that is, soon after the lytic phase) and in B cells after long-term latency (Flano et al., 2003).

Inflammatory cytokines and behavior in GHV infections

Patients with EBV infections typically report sore throats and respiratory symptoms during the active phase of infection and develop daytime sleepiness and fatigue during both active and latent stages (Vollmer-Conna et al., 2004). Circulating and centrally induced cytokines may mediate some of these symptoms. For example, administration of exogenous IFNγ causes daytime fatigue in humans, (Spath-Schwalbe, Lange, Perras, Fehm, & Born, 2000) and B cells from patients with latent EBV infections express IFNγ whereas cells from noninfected people do not (Jabs, Wagner, Schlenke, & Kirchner, 2000). Circulating levels of the proinflammatory cytokines IL1β and IL6 correlate with the occurrence of symptoms (for example, fever, malaise, pain, fatigue, depressed mood, and inability to concentrate) in patients with active EBV infection (Vollmer-Conna et al., 2004). However, relatively little is known about how changes in

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sleep, core temperature, activity, and other measures of well-being change with respect to the host immune response and disease progression. Overall, immune responses in humans have not yet been linked strongly to behavior or illness across the time course of active and latent phases of EBV infection. However, performing such studies in mice with MuGHV infections could provide insights into the pathogenesis of fatigue and other sickness behaviors that can be debilitating to humans.

Although cytokines are normally part of the host mechanisms for controlling the virus, they also can promote both the spread of GHV and the survival of infected cells. During the lytic phase of EBV infection, the viral envelope component glycoprotein (gp) 350 is the primary modulator of expression of cytokines and their receptors in the host (Jung et al., 2001). gp350 mediates virus absorption and penetration into host cells via the complement receptor CD21 (Jung et al., 2001). A 1-to-1 interaction between CD21 and GHV-infected cells is required for adsorption and viral endocytosis and is the primary determinant of EBV tissue tropism (Tanner, Weis, Fearon, Whang, & Kieff, 1987; Tanner, Whang, Sample, Sears, & Kieff, 1988; Urquiza, Lopez, Patino, Rosas, & Patarroyo, 2005).

Binding of gp350 to CD21 induces binding of NFIL6, an encoding nuclear factor that in turn induces the expression of IL6, nuclear factor κB, and other factors that contribute to IL6 expression (D'Addario, Libermann, Xu, Ahmad, & Menezes, 2001). IL6 stimulates proliferation of B cells and thereby promotes spread of the virus by generating targets for infection. In addition, IL6 inhibits the killing of virally infected cells by natural killer cells (Tanner & Tosato, 1991), further promoting the survival of infected cells. Further, gp350 promotes expression of tumor necrosis factor α, IL8, and

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IL10 in monocytes via nuclear factor κB and related cascades (for example, protein kinase C, phosphoinositide 3 kinase, and tyrosine kinases) (D'Addario et al., 2000). The MuGHV homolog of EBV gp350 is gp150, which is crucial for the stimulation of cytokine responses (Stewart et al., 1996).

Cytokines are pivotal in the modulation of both innate and adaptive immunity during MuGHV infections; their release at different times of infection is summarized in Figure 1. The lytic phase of MuGHV infection is associated with production of IL1α, IL2, IL6, and IL10 in the spleen (Sarawar et al., 1996). IFNγ is vital for clearance of virus from the lung via the induction of cytotoxic T cells, natural killer cells, and macrophages and controls the latent phase by curtailing reactivation (Dutia, Clarke, Allen, & Nash, 1997; Sarawar et al., 1997). IL6 is present in high concentrations in blood during MuGHV infection (Sarawar et al., 1996). IL10 also influences MuGHV pathogenesis. For example, IL-10 knockout mice show reduced viral load in the spleen yet increased splenomegaly (Peacock et al., 2001).

The relationships of cytokines to the development of sickness behaviors (for example, anorexia, fever, fatigue, behavioral depression, hyperalgesia, sleep changes) in mice have not yet been documented for MuGHV infection. Although the immune responses induced by EBV and MuGHV infections have many similarities (Flano et al., 2002), little is known about virus-induced behavioral changes under either condition. Studies on EBV and behavior are complicated by uncertainties regarding the dose of virus received, and the time course of disease development and regression may vary due to environment or genetics of the patient. Therefore, not only is MuGHV a good model for studying interactions between GHV infection and host immune responses, but it may

also be useful as a well-controlled model for studying interactions between immune responses and behavioral symptoms.

Other considerations relevant to MuGHV

The strain of mice used can be an important consideration in the study of MuGHV infections. For example, BALB/c mice show greater induction of chemokines and greater viral gene expression during the lytic phase of the infection than do C57BL/6 mice; infected BALB/c mice also have higher levels of IFNγ in lung (Weinberg, Lutzke, Alfinito, & Rochford, 2004). The greater chemokine production by BALB/c mice could promote recruitment of leukocyte target cells to the lungs, thereby augmenting viral replication and increasing susceptibility to infection in these mice. However, the latent viral load in spleen was similar in both strains at 15 d after inoculation, suggesting that the establishment of latency was similar in the 2 strains (Weinberg et al., 2004). These strain differences are likely to be caused by genetic and inherent immune response differences in these mouse strains, such as the differences in IFNγ protein response.

Both the route of administration and the dose of virus used to inoculate mice with MuGHV vary across studies. However, the frequency of in vitro reactivation in splenocytes was the same across a 10^7-fold range of doses administered via intraperitoneal injection and across a 10^4-fold range of doses administered via intranasal administration (Tibbetts et al., 2003b). These findings suggest that the establishment of latency is relatively independent of both the route of administration and infectious dose and that latent infection can be established with extremely low doses of virus.

Differences between MuGHV and EBV

MuGHV and EBV differ in terms of their subgroups, tendency to induce lymphoma, and the nature of CD8+ T cell expansion. Due to genomic organization, EBV is classified as a gamma-1-herpesvirus, or a lymphocryptovirus, whereas MuGHV is classified as a gamma-2-herpesvirus, or a rhadinovirus. More specifically, the MuGHV genome lacks homologs of EBV latency-associated and transforming proteins. Rhadinoviruses also differ from lymphocryptoviruses in their ability to infect both B and T cells (Simas & Efstathiou, 1998). Furthermore, EBV is well-known to cause lymphomas, whereas lymphomas occur only in a low percentage of MuGHV-infected mice, and lymphomas from infected mice contain few MuGHV-infected cells (Sunil-Chandra et al., 1992). MuGHV and EBV also differ in the nature of expanded T cell populations. In EBV infections, CD8+T cells are activated during the acute response to lytic epitopes and are Vβ-specific (Sutkowski, Conrad, Thorley-Lawson, & Huber, 2001). In contrast, the response to MuGHV is unrestricted with regard to major histocompatibility complex haplotype and is not specific for viral epitopes (Flano et al., 2002).

Conclusions

Although MuGHV and EBV differ in several ways, their similarities outweigh their differences and create an opportunity for use of MuGHV as an in vivo model of EBV and other GHVs that elicit debilitating behavioral symptoms such as chronic fatigue and sleep problems. Like EBV, MuGHV causes a life-long infection that is associated with various disease conditions. Both viruses impede host immune processes and evade immune surveillance. Because of numerous impediments and limitations to human GHV

research, the availability of this in vivo murine GHV infection will facilitate elucidation of mechanisms that underlie and link virus reactivation, cytokine release, and clinical symptoms such as chronic fatigue. MuGHV offers an effective in vivo tool for surmounting the hurdles inherent in human GHV research and provides an excellent model to study the behavioral effects in response to immune challenge by GHVs, such as EBV.

Table 2.1 Partial list of mammalian gammaherpesviruses (limited list)

Lymphocryptoviruses (gamma 1)	Rhadinoviruses (gamma 2)
Rhesus lymphocryptoviruses	Herpesvirus saimiri (HVS)
Epstein-Barr virus (HHV4)	Kaposi sarcoma-associated herpesvirus (KSHV or HHV8)
Herpesvirus papio (of baboons)	Rhesus monkey rhadinovirus (RRV) Equine herpesvirus 2 Murine herpesvirus 68

Table 2.2 Diseases associated with EBV infection[*]

Primary infections	Chronic infections and lymphoproliferative disorders	Lymphomas and leukemias	Carcinomas
Infectious mononucleosis	X-linked recessive lymphoproliferative disorder (Duncan disease)	Burkitt lymphoma	Nasopharyngeal carcinoma
EB virus-associated hemophagocytic syndrome (EB-VAHS)	Lymphoproliferative disorders in immunocompromised hosts	Lymphomas in immuno-compromised hosts	Gastric cancer
Gianotti-Crosti syndrome	Hemophagocytic lymphohistiocytosis	Pyothorax-associated lymphoma	Salivary gland cancer
	Chronic active EBV infection	Primary effusion lymphoma (coinfection with HHV8)	Oral hairy leukoplakia
	Hypersensitivity to mosquito bites	Methotrexate-associated lymphoma	
	Hydroa vacciniforme	Lymphomatoid granulomatosis	
		Extranodal NK/T cell lymphoma, nasal type	
		Hydroa vacciniforme-like lymphoma	
		Aggressive NK/T ell lymphoma/leukemia	
		Chronic NK cell leukemia Hodgkin lymphoma	
		Angioimmunoblastic T cell lymphoma (bystander EBV+ cells)	

*Adapted from reference 29
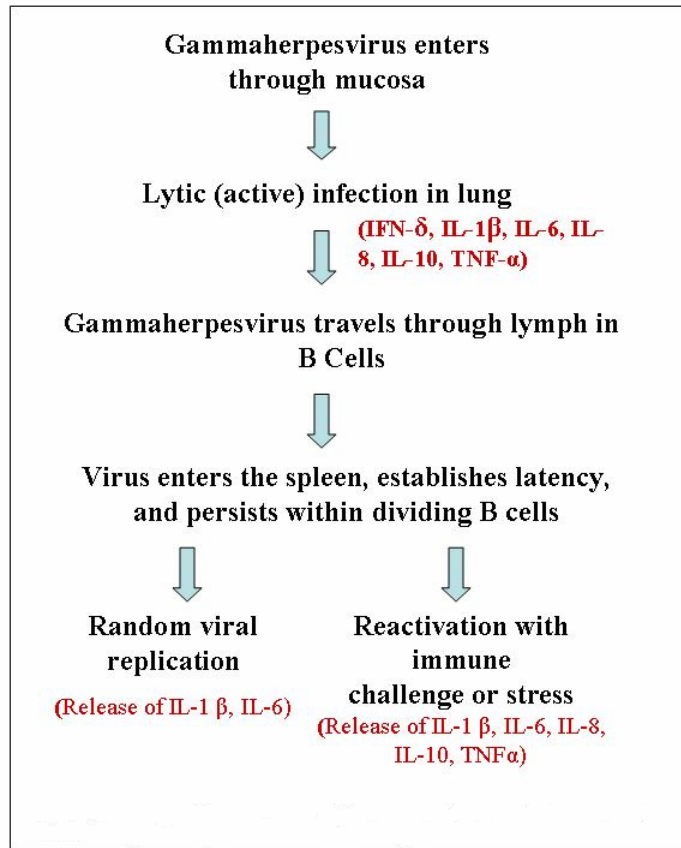
Figure 2.1. Schematic representation of infection with gammaherpesvirus and various cytokines produced during these stages.

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EFFECTS OF INFECTION WITH MURINE GAMMAHERPESVIRUS ON BEHAVIOR OF C57BL/6J MICE: A MOUSE MODEL OF POST-VIRAL CHRONIC FATIGUE

Abstract

INTRODUCTION: Human gammaherpesvirus (GHV) infections, such as with Epstein Barr Virus (EBV) are associated with chronic fatigue. Immune responses to EBV include elevated concentrations of cytokines known to regulate sleep and induce fatigue. Murine GHV-68 (MuGHV) has been used as a mouse model of effects of EBV infection on immune function. However, the impact of infection with MuGHV on behavior of mice has never been studied. We hypothesized that MuGHV infection induces fatigue and alter sleep of mice. Furthermore, we hypothesized that chronic infection with MuGHV exacerbates responses to subsequent challenge.

METHODS: Mice were maintained on a 12:12 h light:dark cycle at 29 °C. Animals were surgically implanted under isoflurane anesthesia with transmitters to record core body temperature, activity, and the EEG and were housed in cages containing a running wheel and infrared detector. Mice were administered vehicle intranasally (i.n.) at dark onset and control recordings were made for 4 days. Mice were then inoculated i.n. at dark onset with 400 PFU (n=13) or 40,000 PFU (n=8) of MuGHV and recordings

made for 30 days post-inoculation. After this 30 day period, they were injected interperitoneally (i.p.) at dark onset with vehicle and 24 h of control recordings. After this control period, they were then subjected to a secondary immune challenge in the form of an IP injection of 10 μg lipopolysaccharide (LPS). Recordings continued for 5 days.

RESULTS: Infection of mice with MuGHV decreased wheel running, core body temperature, food intake and body weight in dose-specific patterns. Although sleep of mice inoculated with 400 PFU MuGHV was not altered, mice inoculated with 40,000 PFU MuGHV spent more time in NREM sleep and less time in wakefulness, changes that coincided with the peak of active viral replication. The impact of secondary immune challenge with LPS on sleep and body temperature differed with dose of inoculation, with the major effect being one of prolonged duration of hypothermia and increases in NREM sleep in viral-inoculated mice.

CONCLUSIONS: These results indicate that mice infected with MuGHV become fatigued as evidenced by reductions in activity. In addition, sleep is altered and there are long-term effects on thermoregulatory mechanisms. These results also suggest that the ability of the immune system to respond normally to subsequent challenge is compromised in mice infected with MuGHV. Overall, these studies suggest infection of mice with MuGHV may be a useful model for facets of post-viral chronic fatigue.

Introduction

Chronic fatigue is a widespread, disabling symptom that decreases quality of life and productivity in the workplace (Bombardier & Buchwald, 1996). Chronic fatigue syndrome (CFS) is difficult to study in humans due to the heterogeneous patient population and the variety of triggering events (Afari & Buchwald, 2003; Evans, 1991; Krupp, Mendelson, & Friedman, 1991; Kyle & DeShazo, 1992). Although the etiology of CFS remains elusive, many patients develop symptoms similar to CFS after infection with Epstein-Barr virus (EBV), a gammaherpesvirus (GHV). Numerous studies suggest EBV infection may be central to the pathogenesis of CFS and may perpetuate the chronic state of fatigue (Glaser & Kiecolt-Glaser, 1998; Jones, 1988; Natelson et al., 1994). The hypothesis that infection with EBV is a triggering event for CFS derives, in part, from observations that individuals with CFS have higher antibody titers to EBV (Glaser et al., 2005; Matthews, Lane, & Manu, 1991; Swanink et al., 1995). CFS patients also respond abnormally to secondary immune challenge of lipopolysaccharide (LPS), suggesting abnormal immune responses to subsequent infection (Gaab, et al., 2005). EBV is a ubiquitous human GHV that establishes life-long latency in the host. EBV and other GHVs have two distinct phases, an active infectious phase, and a latent phase (Flano, Woodland, & Blackman, 2002a). GHVs are able to survive for the lifetime of the host because they have evolved complex mechanisms to evade detection by the host immune system. Transmission of EBV can occur through blood products, transplantation, saliva, and sexual activity (Mandell, Bennett, & Dolin, 1995), which makes prevention of the spread of this virus extremely challenging. Data indicate that

95% of all adults have antibodies against EBV (Rickinson & Kieff, 2001; Mandell et al., 1995), yet only a small fraction of adults develop CFS.

Cytokines may play a role in the pathophysiology of CFS because patients have elevated concentrations of interleukin (IL)-1, IL-6 and interferon (IFN)-γ (Hornef, Wagner, Kruse, & Kirchner, 1995). These aforementioned cytokines are implicated in the regulation of multiple central nervous system processes, among them sleep and thermoregulation. Many individuals infected with EBV and CFS patients report nighttime sleep disturbances as well as daytime fatigue (Unger et al., 2004). CFS patients report a high incidence of low-grade fevers, possibly due to alterations in thermoregulatory mechanisms (Fukuda et al., 1994). However objective studies of fatigue, sleep disturbances and temperature alterations in humans have not been widely done due to the difficulty in studying EBV and CFS.

A mouse GHV, MuGHV-68, has been identified that shares many similarities with human EBV (Flano, Woodland, & Blackman, 2002b). Both viruses have an infectious active phase in the lung and establish latency in the spleen. MuGHV is a reliable model for EBV pathogenesis because it is a herpesvirus that has the same route of infection, elicits the same innate and adaptive immune responses, and has similar DNA structure [reviewed (Olivadoti, Toth, Weinberg, & Opp, 2007)]. In addition, MuGHV does not normally cause mortality in mice, which allows assessment of chronic effects of long-term viral infection. In this study we leverage these features of chronic infection with MuGHV-68 to determine the impact of chronic viral infection on sleep-wake behavior, activity and temperature patterns of C57BL/6J mice. We hypothesize that infection with MuGHV alters activity patterns, sleep, and core body temperature in a

manner that resembles facets of human responses to EBV. Furthermore, we hypothesize that the impact of chronic infection on these behaviors and processes depends upon the magnitude of viral load during infection. We also predict that responses to secondary immune challenge of chronically infected mice are exacerbated. We now report that the magnitude of alterations of sleep and activity patterns, body temperature and responses to challenge with LPS during chronic infection depend on viral load.

Methods

Animals

Adult male C57BL/6J mice (22 – 30 g at time of surgery; the Jackson Laboratory, Bar Harbor, ME) were individually housed in microisolator cages and maintained on a 12:12 h light:dark cycle at an ambient temperature of 29 ± 1 ºC. Water and rodent chow (Lab Diet 5001, PMI Nutrition International, Brentwood, MO) were provided *ad libitum*. Food was recorded daily. All procedures were approved by the University of Michigan Committee on Care and Use of Animals in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and Use of Laboratory Animals.

Surgical Procedure

Under isoflurane anesthesia (4% induction, 2% maintenance), a battery-operated telemeter [model # ETA10-F20, Data Sciences International (DSI), St. Paul, MN] was surgically implanted into the peritoneal cavity. Insulated biopotential leads from the transmitter were subcutaneously routed to the skull. The biopotential lead wires were

then attached to two small stainless steel screws (#80 x 1/8 in., Small Parts, Miami Lakes, FL), which were implanted in the skull over the frontal and parietal brain cortices and served as electroencephalographic (EEG) recording electrodes. After surgery the mouse was placed on a heating pad at 37 ºC until it recovered from anesthesia and was ambulatory. Ibuprofen (0.2 mg/mL) was provided in the drinking water as an analgesia beginning 24 h before surgery and continuing for 48 h after surgery (Hays, et al., 2000). A broad-spectrum antibiotic (imipenem, 25 mg/kg subcutaneous) and an injectable analgesic (buprenex, 0.05 mg/kg) were given immediately after surgery. Mice were allowed 21 days to recover from surgery before experiments began. Mice were housed in the recording room during the recovery period to habituate to the recording environment.

Recording Apparatus

The signal emitted by the transmitter was detected by a receiver (RPC-1, DSI) located under each mouse's cage. The signal was then fed to a DSI analog converter (ART Analog-8 CM) and frequency signals converted into EEG and temperature voltages using calibration factors provided by DSI that were specific for each transmitter. The output from the DSI analog converter was then captured by an A/D board (model PCI-3033E, National Instruments, Austin, TX), which digitized the data at 128 HZz with 16-bit precision. The voltage values obtained from the temperature channel of the transmitter were converted to engineering units (°C) by regression using coefficients specific for each transmitter and obtained by calibration with a water bath. This commercially available telemetry system has previously been used to determine sleep-wake behavior of mice (Tang & Sanford, 2002; Morrow & Opp, 2005a; Morrow & Opp,

2005b), and temperature and activity patterns from other small mammals (Herold, Spray, Horn, & Henriksen, 1998). Cage activity was detected using an infrared sensor housed in an observation unit that also contained a camera (BioBserve, GmbH, Bonn, Germany). Thus, mice could be observed during both the light and dark period of the light:dark cycle. Movements detected by the infrared sensor were converted to a voltage output, the magnitude of which was directly related to the magnitude of movements detected. Wheel running was determined by detecting TTL pulses triggered by a switch on the axel of the wheel. All signals (EEG, body temperature, wheel running and cage activity) were stored as binary files until further processing.

During acquisition, the EEG was digitally filtered using Chebyschev filters with 3rd order coefficients into frequency bands as follows: delta, 0.5 to 4.0 Hz; theta, 6.0 to 9.0 Hz; alpha, 12 to 16 Hz. These filtered EEG signals were integrated over 1-s periods, and stored as part of the binary file structure. Arousal state designations were made with 10-s resolution on the basis of visual inspection of the recordings using custom software (ICELUS, M. Opp, University of Michigan) written in LabView for Windows (National Instruments). Determination of arousal state was made on the basis of EEG, body movements, and integrated delta and theta frequency values. Each state was classified as wakefulness, NREM sleep, or REM sleep. Wakefulness was defined on the basis of a low amplitude, mixed frequency (delta $\cong$ theta) EEG accompanied by body movements. Increases in body temperature during wakefulness are associated with activity. NREM sleep was identified by increased absolute EEG amplitude, integrated values for the delta frequency band greater than those for theta, and lack of body movements. Body temperature declines upon entry into NREM sleep until it reaches a regulated asymptote.

REM sleep was characterized by a low amplitude EEG, with integrated values for the delta frequency band less than those for the theta frequency band. Epochs containing either movement artifacts or electrical noise were tagged and excluded from subsequent spectral analyses. The raw, non-integrated EEG signals were also processed using fast fourier transforms (FFT) to yield power spectra between 0.5 and 40 Hz in 0.5 Hz frequency bins. These spectra were computed from the five consecutive 2-s EEG segments comprising the 10-s epoch. These five spectra were averaged to produce one spectra for the epoch, which was matched to state to provide state-specific power spectra. Measures of sleep consolidation / fragmentation were made based upon determination of the number of transitions from one arousal state to the next.

Experimental Protocols

Viral Preparation and Inoculation Procedure

Viral stock of MuGHV-68 (8.2×10^5 PFU/mL, Blackman Lab, Trudeau Institute) was stored at -80º C and thawed just prior to use. To achieve a 400 PFU dose the stock was diluted to by adding 170 μL of viral stock to 10 mL of Hank's Balanced Salt Solution (HBSS). 50 μL of straight stock was used to inoculate mice in the 40,000 PFU dose group. Mice were lightly anesthetized with isoflurane and held upright by the scruff of the neck while 30 – 50 μl of fluid were placed into the nostrils (i.n.) with a micropipette. Approximately half of the liquid was injected into each nostril and mice were observed until waking from the anesthesia.

Experiment I: Impact of MuGHV infection on sleep, activity and body temperature.

After recovery from surgery, mice were placed in microisolator cages equipped with running wheels and were given two weeks for adaptation to use of the wheel. After adaptation, mice were inoculated at dark onset with vehicle (HBSS) (30 μL for 400 PFU, 50 μL for 40,000 PFU). After i.n. administration of vehicle, control recordings of EEG, core body temperature, wheel running and cage activity were obtained for four consecutive days (see Figure 3.1). After control recordings, mice were inoculated at dark onset with either 30 μl vehicle containing 400 PFU of MuGHV (n=13) or with 50 μl vehicle containing 40,000 PFU (n=8) of MuGHV. Recordings continued for 30 consecutive days after MuGHV inoculation. Husbandry was done on day 12 after viral inoculation, and mice were weighed daily. To determine daily food consumption, pre-weighed portions of mouse chow were placed in the cage each day and uneaten chow was removed and weighed. After inoculation of mice with MuGHV all activities were conducted under Biohazard Safety Level 2 conditions.

Experiment II: Lipopolysaccharide (LPS)-induced alterations in sleep, activity and body temperature of chronically infected mice.

On day 34 post-inoculation, infected animals were given intraperitoneal (i.p.) injection of 0.2 mL pyrogen-free saline (vehicle) and recordings obtained for 24 h. Infected mice were subsequently injected i.p. with10 μg of LPS (*Escherichia coli* serotype O111:B4; Sigma-Aldrich, St. Louis, MO) and recordings continued for 5 days.

Experiment III: Determination of viral load during infection with MuGHV.

Mice were kept in the same conditions as above and inoculated with either vehicle or with 40,000 PFU of MuGHV. Mice were sacrificed four days after vehicle administration (n=8), or 5 days (n=6), 9 days (n=6) or 26 days (n=8) after inoculation with MuGHV. Lung and spleen were removed and flash-frozen in liquid nitrogen and stored at -80 °C. DNA extracted from frozen tissue samples (DNeasy Kits, Qiagen, Valencia, CA) was used to determine viral load by quantitative PCR (Hulse, Kunkler, Fedynyshyn, & Kraig, 2004). Viral load was quantified in spleen and lung of infected mice by real-time PCR using the ABI Prism 7700Sequence Detector (PE Applied Biosystems) to detect a 70bp region of the MuGHV-68 gB gene: forward 5'-GGCCCAAATTCAATTTGCCT-3'; reverse 5'-CCCTGGACAACTCCTCAAGC-3'; probe (5') 6-(FAM)-ACAAGCTGACCACCAGCGTCAACAAC-(TAMRA)(3') where FAM is a reporter dye and TAMRA is a quencher dye. Standard curves were generated using known amounts of plasmid containing gB PCR fragment. The DNA copy number was calculated from the molecular weight of the DNA construct of Avogadro's number. One hundred nanograms of DNA extracted from each mouse spleen was used per reaction (Weinberg, et al., 2004).

Statistical Analyses

Statistical analyses were performed using SPSS v. 13.0 for Windows. Data were evaluated in 6 and 12 h time blocks using linear mixed models analysis with a Bonferroni correction. The amount of time spent in vigilance states, core body temperature, wheel running, cage activity, transitions from one state to another, and delta power during NREM sleep were the dependent variables and manipulation (vehicle, MuGHV) was the

independent variable. An alpha level of $p < 0.05$ was accepted for all statistical tests as indicating significant departures from control values.

Results

Experiment I: Impact of MuGHV infection on sleep, activity and body temperature.

Activity

Wheel running activity of mice inoculated with 400 PFU MuGHV progressively declined in the late portion of the dark phase (hours $7 - 12$, $p < 0.05$, Figure 3.2) during days $14 - 30$ post-inoculation.   Wheel running of these mice also decreased during the late portion of the light period throughout the entire course of the infection compared to vehicle control ($p < 0.05$ Figure 3.2). Wheel running of mice inoculated with 40,000 PFU of MuGHV was decreased during both dark and light phases throughout the entire recording period ($p < 0.01$ dark period, $p < 0.001$ light period, Figure 3.2). General cage activity of mice inoculated with either the 400 PFU or 40,000 PFU dose of MuGHV was not altered at any time throughout the recording protocol (data not shown).

Food intake and body weights

Food intake decreased significantly during days $8 - 10$ ($p < 0.05$), and body weight dropped during days $7 - 14$ after inoculation with 40,000 PFU of MuGHV ($p < 0.05$, Figure 3.4).   Food intake and body weights of mice were not altered after inoculation with 400 PFU MuGHV (Figure 3.5).

Body temperature

Mice inoculated with 400 PFU MuGHV responded with hypothermia that progressed throughout the course of infection and deviated statistically between post-inoculation at various time points throughout the recording period (Figure 3.3). The largest drops in body temperature occurred during the second half of the dark period [hours 7 – 12 (post-inoculation days 14 – 30, $p<0.05$, Figure 3.3)], and the second half of the light period [hours 13 – 18 (post-inoculation days 14 – 20, $p<0.05$, Figure 3.3)], Mice inoculated with 40,000 PFU had a significant hypothermia on day 9 post-infection compared to vehicle ($p<0.01$ Figure 3.2) during the late portion of the dark period.

Sleep-wake behavior

Visual scoring of 24 h records from days 1, 3, 5, 7, 9, 24, 26, 28, and 30 post-inoculation was completed for mice inoculated with 400 PFU, and scoring was completed on days 3,5,7-14, and 26 for mice inoculated with 40,000 PFU. Scoring results from mice infected with 400 PFU indicated that sleep-wake behavior of mice was not altered (data not shown). In contrast, sleep-wake behavior of mice inoculated with 40,000 PFU of MuGHV was altered for prolonged periods. On day 7 after inoculation NREM sleep was decreased, however between days 8 – 11 post-inoculation with this dose of MuGHV, NREM sleep was increased ($p < 0.001$; Figure 3.6). The increase in NREM sleep occurred primarily during the dark period of days 8-11. Effects of 40,000 PFU of MuGHV on REM sleep were apparent during the dark periods of days 8, 9 and 10 post-inoculation with decreases in NREM sleep on these days after inoculation ($P<0.05$; Figure 3.6). Sleep of mice infected with 40,000 PFU of MuGHV was fragmented during the light period of day 7 and during the dark period of days 8 and 9 ($p<0.05$), as

evidenced by significant increases in the number of transitions from one state to another. Significant decreases in wakefulness during the dark period of day 9 were also observed (p<0.05). Sleep-wake behavior on days 5 and 26 after inoculation with 40,000 MuGHV was not altered (Figure 3.6). Delta power during NREM sleep did not significantly change after inoculation with either dose of MuGHV (data not shown).

Experiment II: LPS-induced alterations in sleep, activity and body temperature of chronically infected mice.

Food intake and body weight

There were no significant differences in food intake or body weights of mice inoculated with 400 PFU of MuGHV and subsequently challenged with LPS compared to vehicle control. Although food intake of mice inoculated with 40,000 PFU of MuGHV decreased during day 4 after LPS administration (p<0.05), drops in body weight achieved statistical significance only during the first two days after LPS administration (data not shown, p<0.01). The impact of LPS on food intake and body weights of mice inoculated with MuGHV differed depending on the dose of viral inoculation; food intake on day 3 and body weights on days 1 and 3 post-LPS administration of mice inoculated with 40,000 PFU of MuGHV were significantly less than those of mice inoculated with 400 PFU of MuGHV (data not shown).

Body temperature

Uninfected C57BL/6J mice injected i.p. with LPS developed hypothermia that lasted for 7 hours (p=0.01, Figure 3.7). This period of hypothermia was followed by

fever during the subsequent light period, 12 h after LPS injection (p<0.001). These results are from previously published data (Morrow, Opp, 2005) that has been re-analyzed. Hours 24-48 after LPS were newly analyzed from this previously published data set. Hypothermic responses to LPS of mice inoculated with 400 PFU of MuGHV were prolonged (Figure 3.8); these animals were hypothermic during the dark periods of days 1 and 2 post-inoculation (hours 1- 12 and 24 – 36, p<0.01, nadir magnitude of -2.2 ± 0.6 ºC). The hypothermic response to LPS in these animals was still apparent during the late portion of the dark periods of days 3 – 5 (data not shown). Similar to uninfected C57BL/6J mice, the temperature responses of mice inoculated with 40,000 PFU of MuGHV to LPS consisted of an initial 6-h febrile response (peak magnitude of 1.3 ± 0.5 ºC) followed by a hypothermic response that lasted 12 h (nadir magnitude of -1.78 ± 0.25 ºC, Figure 3.9). However, in contrast to uninfected C57BL/6J mice and similar to mice inoculated with 400 PFU of MuGHV (Figure 3.8), mice inoculated with 40,000 PFU of MuGHV exhibited hypothermic responses during the second and third dark periods (hours 24 – 36 and 48 – 60) post-injection (Figure 3.9). The hypothermic responses of mice inoculated with either 400 PFU or with 40,000 PFU of MuGHV were of greater magnitude, and of longer duration than observed in uninfected C57BL/6J mice.

Sleep-wake behavior

Injection of LPS at dark onset into uninfected C57BL/6J mice increased NREM sleep and suppressed REM sleep during post-injection hours 1 – 12 (Figure 3.7). However, unlike responses of uninfected mice, the LPS-induced increase in NREM sleep of C57BL/6J mice inoculated with 400 PFU of MuGHV was prolonged and apparent

during post-injection hours 1 – 12 and during the subsequent dark period (post-injection 24 – 36) ($p<0.05$, Figure 3.8). After this period during which NREM sleep was increased, NREM sleep of mice inoculated with 400 PFU of MuGHV and injected with LPS was reduced during the light periods of days 3 – 5 post-injection (data not shown). REM sleep of these animals was reduced during most of the light periods on post-injection days 3 – 5 ($p< 0.05$, Figure 3.8). Sleep of mice inoculated with 400 PFU of MuGHV and then injected with LPS was fragmented for 36 hours post-injection ($p<0.001$, Figure 3.8) as well as during the light period of days 3, 4 and 5 (data not shown). Amount of time mice inoculated with 400 PFU of MuGHV spent awake mirrored increases in NREM sleep, being reduced during the first 12 h after LPS injection ($p<0.01$) and increased during most portions of the light period on post-injection days 3 – 5 ($p<0.001$, Figure 3.8). NREM sleep of mice inoculated with 40,000 PFU of MuGHV was increased hours 1 – 12 and 24 – 30 post LPS injection ($p < 0.001$), whereas REM sleep was reduced during post-injection hours 12 – 18 and 24 – 32 ($p < 0.001$, Figure 3.9). Sleep of these mice was fragmented during the first 18 h after LPS ($p < 0.001$, Figure 3.9). Delta power during NREM sleep of mice inoculated with 400 PFU was decreased during the light period 12 hours after LPS injection whereas delta power during NREM sleep of mice inoculated with 40,000 PFU MuGHV was not significantly altered (Figure 3.9).

MuGHV infection had differential impact on LPS-induced alterations in sleep-wake behavior of mice depending on the dose used (Figure 3.10). NREM sleep during the 12 h dark period after injection of LPS of mice inoculated with either 400 PFU or with 40,000 PFU was more fragmented than NREM sleep of uninfected C57BL/6J mice

during comparable periods (p<0.05). In addition, the effect of LPS on REM sleep of mice inoculated with MuGHV differed from that of uninfected C57BL/6J mice. REM sleep of mice inoculated with 400 PFU of MuGHV was not altered by LPS, whereas REM sleep of mice inoculated with 40,000 PFU of MuGHV was suppressed by LPS injection at dark onset during the subsequent light period, postinjection hours 13 − 24 (p<0.05). Delta power during NREM sleep of mice inoculated with 400 PFU of MuGHV and then injected with LPS was reduced to a greater extent during postinjection hours 13 − 18 than that observed in mice inoculated with 40,000 PFU of MuGHV (p<0.05).

Experiment III: Determination of viral load during infection with MuGHV.

Viral DNA was detected in the lungs and spleens of mice inoculated with MuGHV (Figure 3.11). Viral DNA was not detected in control mice. Lung viral load was highest at 5 days post-infection and almost absent on day 26 after both doses of infection. In the spleen, viral load peaked on day 9 post-inoculation and remained detectable at days 26 after inoculation.

Discussion

Our results demonstrate that mice become progressively fatigued throughout the course of chronic viral infection. The temporal characteristics of altered activity, sleep-wake behavior and thermoregulation coincide with transitions from active infection to latency. Furthermore, the magnitude of these changes in complex behavior is dependent upon the dose of virus in the inoculant, i.e., presumed viral load. Of particular interest, fatigue in these mice, as operationally defined as a reduction in activity, is

limited to voluntary activity (wheel running) and not obligatory activity (general cage activity associated with feeding, drinking, grooming, etc.). These results are reminiscent of the diagnostic criteria of CFS in humans; voluntary activities are curtailed while essential activities such as working or taking care of a family are largely maintained (Rakib et al., 2005). Patients suffering from CFS due to different etiologies also differ in the extent of their immune perturbations; patients in which CFS onset occurred after an infection have more extensive alterations in immune function than normal controls (Racciatti, Vecchiet, Ceccomancini, Ricci, & Pizzigallo, 2001).

Gammaherpesviruses (GHV's), such as Epstein-Barr virus (EBV), have been linked to the pathology of CFS (Glaser et al., 2005). Whole blood cultures from individuals infected with EBV have abnormal responses to LPS stimulation compared to EBV-negative individuals, with increased stimulated concentrations of IL-1, TNF-a and IL- 6, and similar findings have been found in patients with CFS as well (Hornef et al., 1995).

Although there is much that can be learned from *in vitro* studies of whole blood cultures from individuals infected with EBV or suffering from CFS, there are limitations to these types of studies. Foremost among these limitations is the inability to directly relate findings obtained by these *in vitro* methods to the impact of chronic infection on daily life. It is difficult to study EBV infection, post-viral chronic fatigue, and effects of subsequent immune challenge in humans due to the heterogeneity of the patient population (age, stage of infection, variable gene expression during latency, unpredictability of fatigue trigger, and the time of infection), and subject sampling methods (Glaser et al., 2005). In addition, ratings of fatigue are assessed through self-

report, which introduces a large component of subjectivity into the results (Hossain et al., 2005). Most mechanistic studies have emphasized immune responses, and not the relationships between immune responses and behavior. Moghaddam et al., (1997) proposed using a lymphocryptovirus in rhesus monkeys, that elicits similar immunological responses, as an immunological model of EBV, however this model is neither economical nor practical for laboratory use, and also does not measure behavioral responses during infection. MuGHV is a model for human infection with EBV that shares structural and biological properties with EBV, infects mice by the same route as EBV in humans, and elicits a similar innate and adaptive immune responses [reviewed in (Olivadoti et al., 2007; Flano et al., 2002b)]. In addition, because MuGHV does not normally cause mortality in mice, it is possible to assess the impact of chronic long-term viral infection. The MuGHV model of EBV infection has been widely used to determine the impact of chronic infection with this virus on immune function. We extend this model to include effects of chronic infection with this virus on CNS function. Activity patterns, sleep-wake behavior, and thermoregulation are altered in mice throughout the course of infection with MuGHV in a manner that mimics aspects of EBV infection on daytime activity and nighttime sleep of humans.

Many patients with CFS or individuals infected with EBV report disrupted nighttime sleep, including problems of sleep maintenance (Rakib et al., 2005; Fossey et al., 2004). Sleep-wake behavior of mice inoculated with MuGHV is altered. The most dramatic alterations in sleep-wake behavior are the increases in NREM sleep and reductions in REM sleep that occur at times that parallel peak viral replication in the lung (Flano, Kim, Moore, Woodland, & Blackman, 2003). In addition, sleep of mice

inoculated with MuGHV is fragmented during these periods of altered behavior. These alterations in sleep of mice inoculated with MuGHV, in some respects, mimic the disruptions to nighttime sleep often reported in patients with CFS or EBV infection. Because the disruptions in sleep of infected mice are most apparent during peak of active infection in the lung, these results also suggest that virus-induced alterations in arousal state are specific to the peak of active infection.

Immune function of patients with CFS has been widely investigated. Although results vary between studies, common findings include impairment of cell-mediated immunity (Klimas, Salvato, Morgan, & Fletcher, 1990; Siegel et al., 2006), chronic immune activation [reviewed in (Devanur & Kerr, 2006)], and elevated concentrations of cytokines (Cannon et al., 1999). In addition, many in vitro studies demonstrate that immune responses of samples from CFS patients differ from those of healthy controls in many aspects. For example, Visser et al. (Visser et al., 2001) report significant differences in cytokine production in response to LPS stimulation of whole blood cultures from CFS patients. LPS stimulation of whole blood cultures from CFS patients also results in increased IgM and IgA (Hornef, et al. 1995; Maes, Mihaylova, & Leunis, 2007), and stimulated production of cytokines such as IL-6 and TNF-α that correlate to fatigue ratings in these subjects (Gaab et al., 2005).

Changes in sleep-wake behavior during infection with MuGHV may be mediated by virus-induced alterations in concentrations and/or profiles of cytokine either in brain or in the periphery. We hypothesize that increased NREM sleep during days 7 – 14 post-inoculation may be driven, in part, by elevated concentrations of IL-1, TNF-α, and/or IL-6. IL-1β and TNF-α are involved in the regulation of normal, spontaneous sleep in the

absence of pathophysiology (Opp, 2005). It is hypothesized that the alterations in sleep in response to immune challenge are due to amplification of these normal physiological processes (Krueger et al., 1994). Body weight and food intake are also significantly altered by a higher dose of viral infection, and these changes coincided with previous reports of peak viral replication in the lung (Flano, et al., 2002). Long-term hypothermia in infected mice was not completely explained by decreases in activity, which signifies that MuGHV affects thermoregulatory mechanisms. Changes in thermoregulatory mechanisms are also noted in patients with EBV and CFS, however the functional significance of hypothermia in mice is largely debated, and thus can only be compared hesitantly to humans.

These results also demonstrate the relative response to infection in behaviors that are expressed according to either the stage of the virus or the presence of chronic viral infection itself, suggesting more than one mechanism of these responses is at work in changing specific behavioral reactions to infection. Understanding how these behaviors coincide with initial contraction and course of infection is important in understanding the human EBV infection in concordance with immunological parameters.

Our results also demonstrate abnormal responses of infected mice to secondary immune challenge, reflected in sleep and temperature measures that suggest changes in the immune system of chronically infected mice, potentially due to latent infection. In addition, the initial dose of virus has an effect on results of secondary immune challenge, suggesting that there are both short-term changes, such as changes in activity, sleep and temperature, as well as long-term immune changes from infection that are dose-specific.

Conclusions

Previous studies have shown differences in immune reaction to MuGHV in different strains of mice (Weinberg, Lutzke, Alfinito, & Rochford, 2004), thus more studies are needed to understand the impact of genetic variation on resulting behaviors due to infection. Additional studies can also help explain the impact of physical or psychological stress on behavioral responses to viral infection to more accurately understand the mechanisms of post-viral fatigue.

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        21 DAYS                                    30 DAYS

  ┌─────────────────────┬────────┬──────────┬─────────────────────────────────┬──────┬──────┐
  │ Surgery and Recovery │ HABIT. │  HBSS    │                Virus            │ PFS  │ LPS  │
  │                      │        │ Vehicle  │  Active                  Latent │      │      │
  └─────────────────────┴────────┴──────────┴─────────────────────────────────┴──────┴──────┘
  -35                    -14      0          4                            34    35    40
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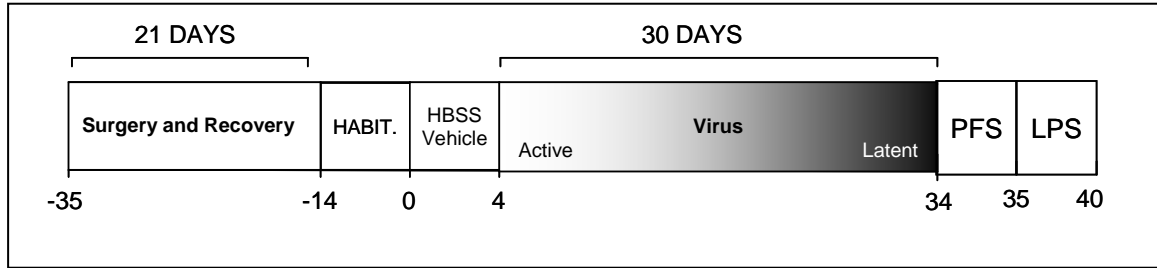
Figure 3.1: Graphic depiction of the timeline used for the protocol in this study. Mice (n=21 total) were implanted with a telemeter and recovered for 21 days. A two-week period was allotted for habituation to the presence of a running wheel. Mice were then given $30 - 50$ µl vehicle intranasally with (Hank's Balanced Salt Solution, HBSS) and recordings were made for 4 days. Mice were then inoculated with either 400 PFU or 40,000 PFU MuGHV and recordings continued for 30 days. On day 34 post MuGHV inoculation, mice were injected intraperitoneally with pyrogen-free saline (PFS) and 24 h control recordings were made. Then mice were injected IP with 10 µg of lipopolysaccharide (LPS) and recordings continued for an additional 5 days.
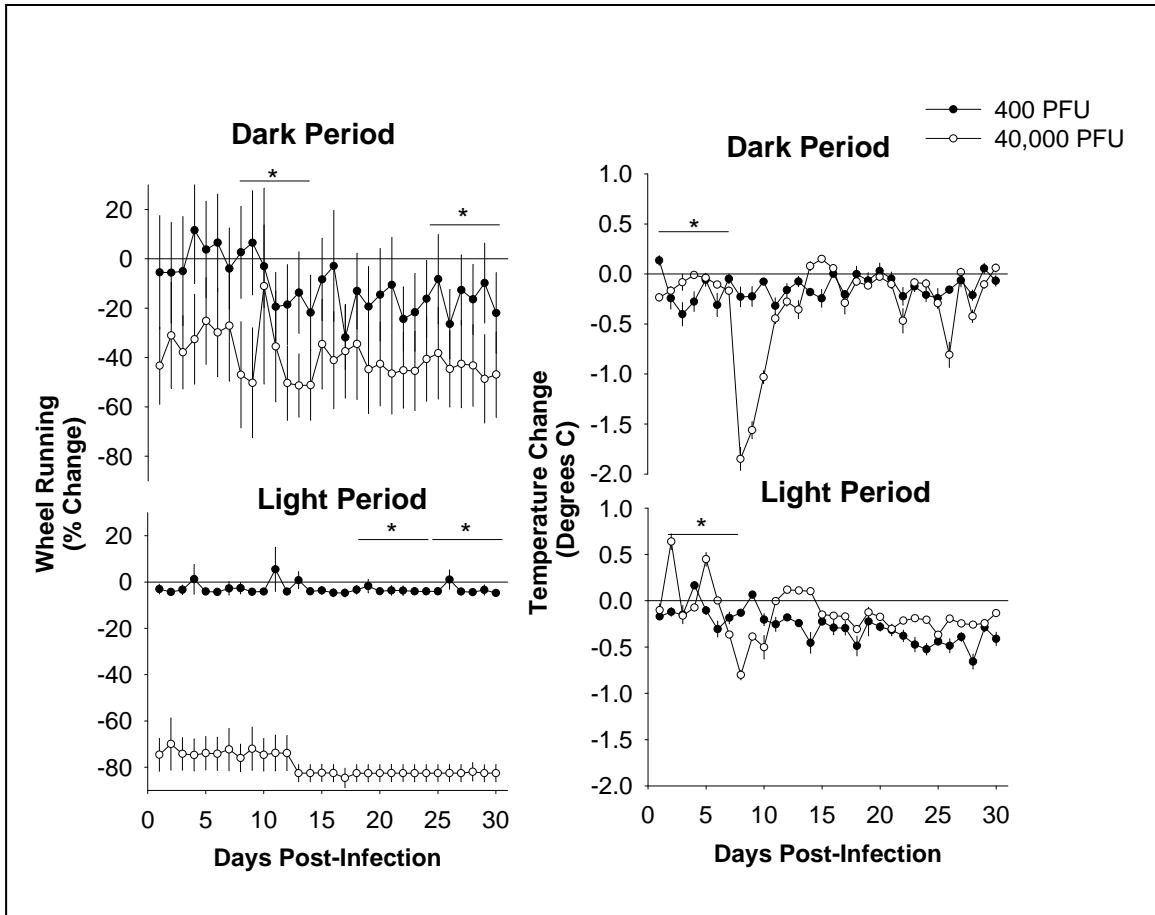
Figure 3.2: Changes in wheel running and core body temperature of mice inoculated with either 400 PFU (n=13, open circles) or with 40,000 PFU (n=8, closed circles) of MuGHV. Values are percent change (wheel running) or degrees difference (temperature) from values obtained after administration of vehicle, represented by the zero line. Error bars are ± SEM. Asterisks (*) indicate significant differences between doses.
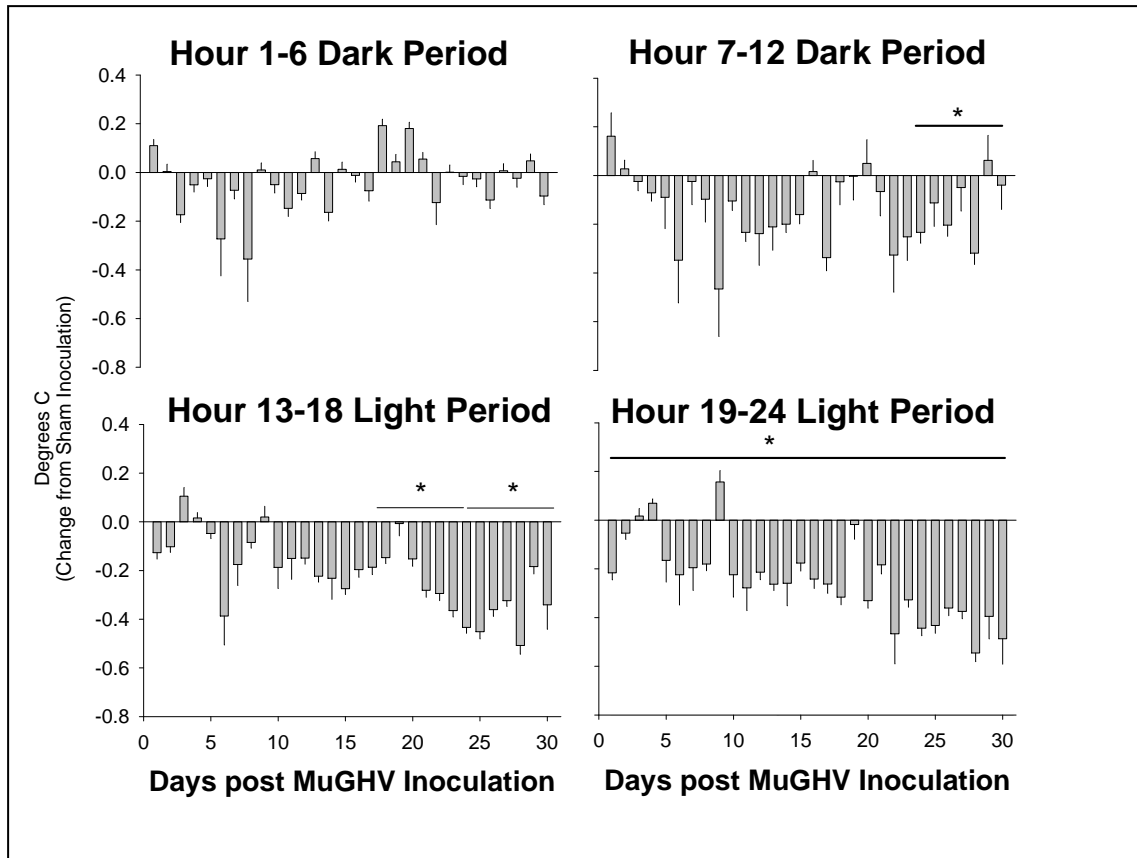
Figure 3.3: Impact of infection with 400 PFU MuGHV on core body temperature in C57BL/6J mice. Values are changes from vehicle (zero line) ± SEM in temperature of infected mice (n=13) over 6 hour periods. Asterisks (*) indicate significant changes from vehicle. Infected mice had significant and chronic decreases in core body temperature during the second half of the dark period during days 21-30 ($p<0.05$) and during the first half of the light period during days 14-30 ($p<0.05$) as well as during all 30 days during the late portion of the light period ($p<0.05$).
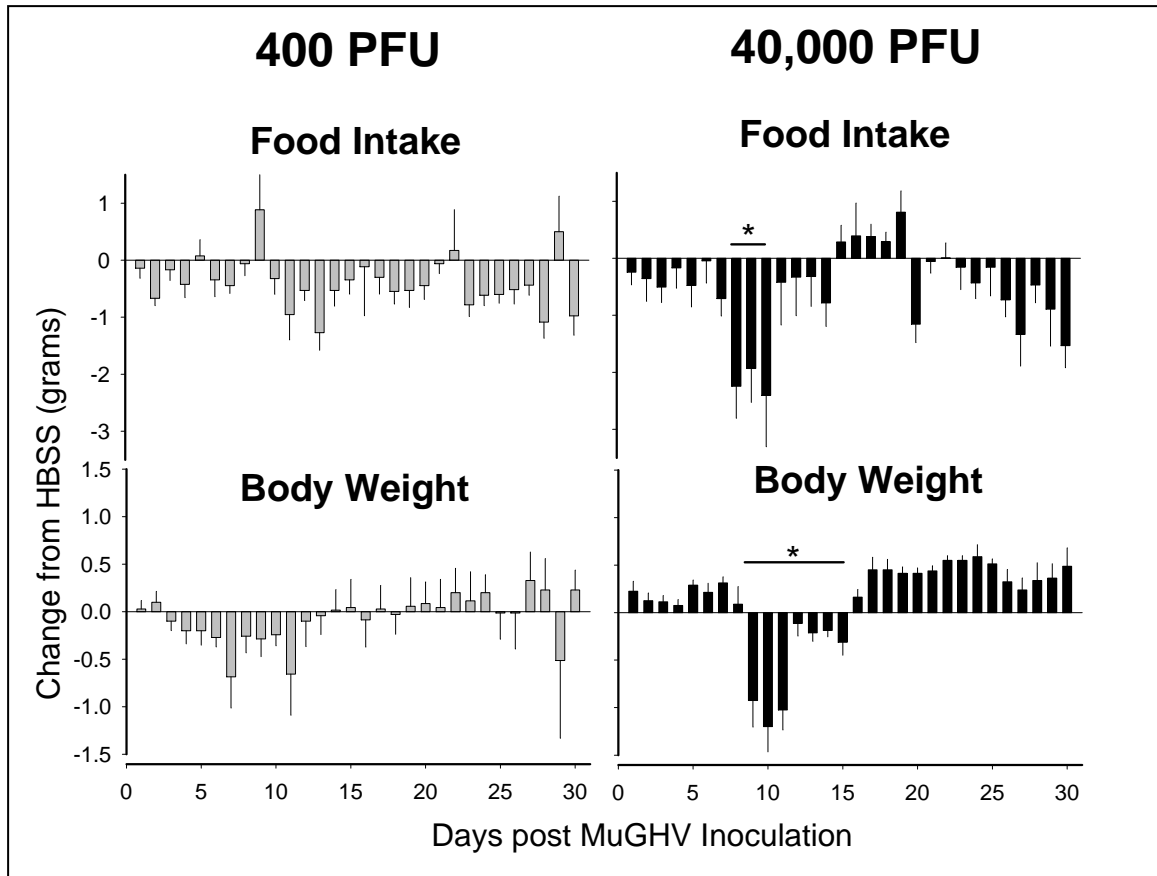
Figure 3.4: Impact of 400 PFU (n=13, grey bars) or 40,000 PFU (n=8, black bars) MuGHV infection on food intake and body weight in C57BL/6J mice. Values are expressed as difference from vehicle body weight and food intake ± SEM. Asterisk (*) indicates significant difference from vehicle ($p < 0.05$). Mice infected with 40,000 PFU had significant decreases in body weight during days 8-14 after inoculation ($p < 0.05$).
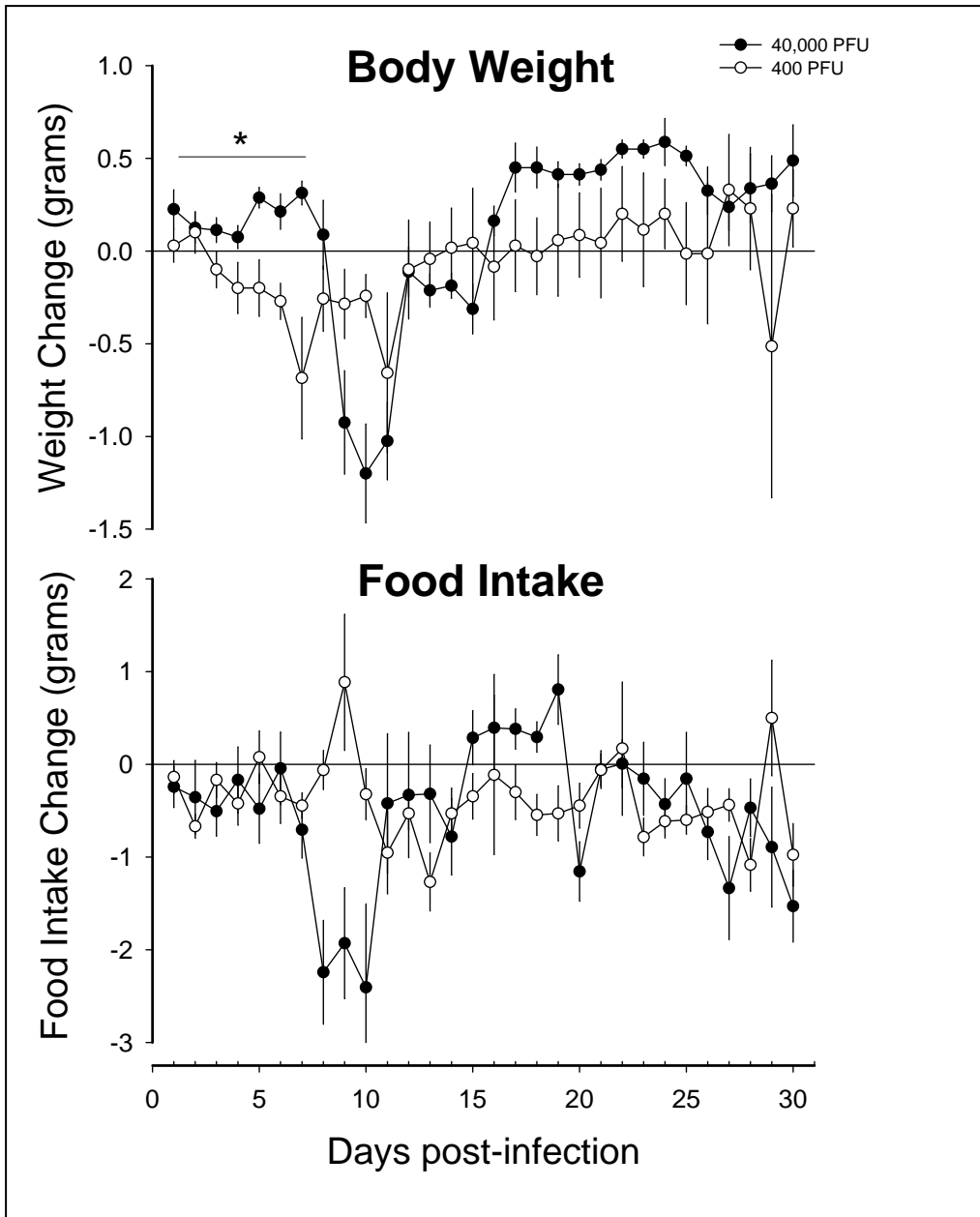
Figure 3.5: Dose comparison of body weight and food intake changes during chronic viral infection with 400 PFU (n=13, open circles) or 40,000 PFU (n=8, closed circles). Values are differences from HBSS for each dose (zero line) ± SEM. Infected mice had significant differences in body weight during the first 7 days after inoculation ($p<0.05$).
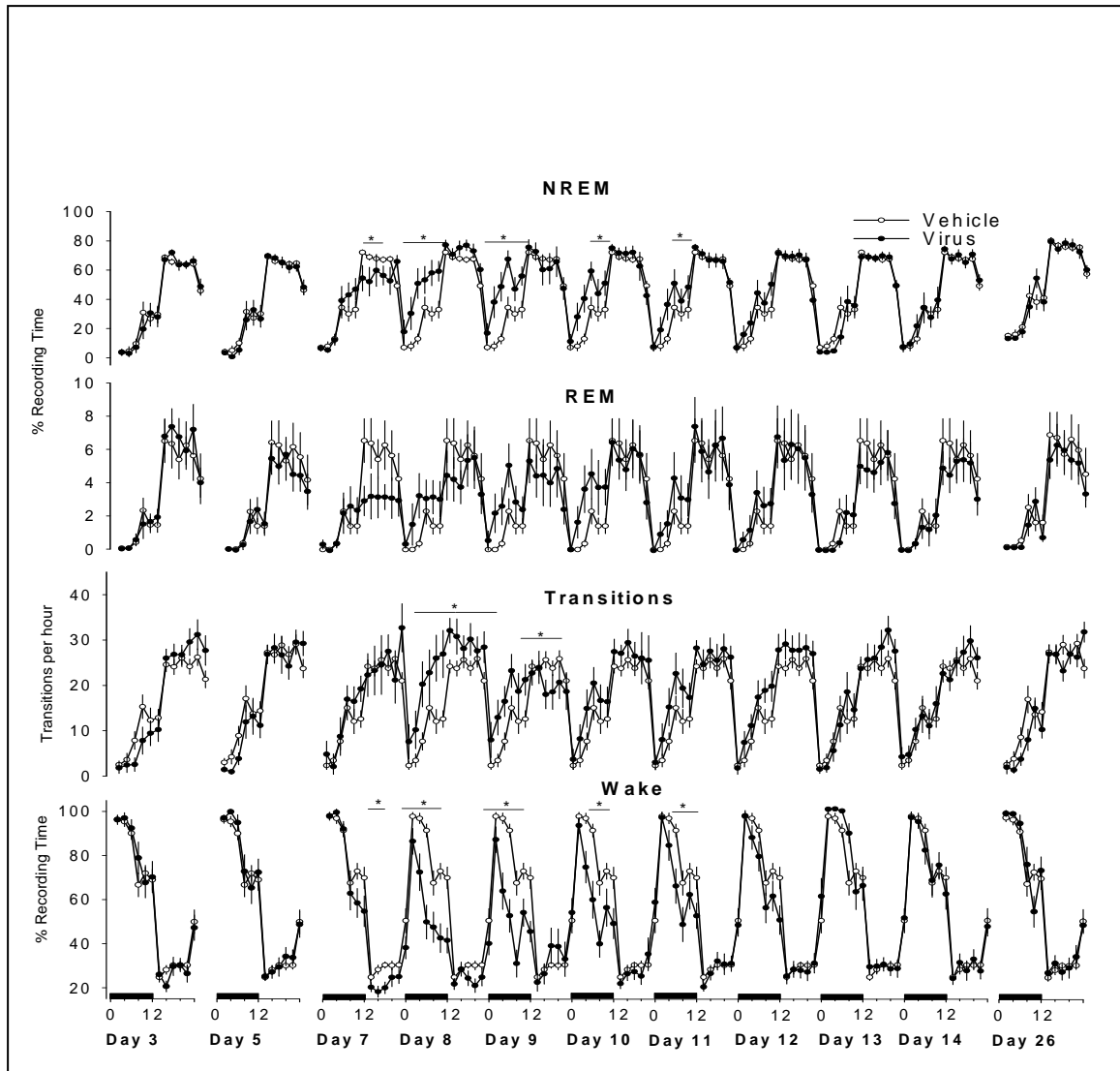
Figure 3.6: Impact of infection with 40,000 PFU MuGHV (n=8, filled circles) on sleep parameters in C57BL/6J mice compared to vehicle (empty circles). Black bars signify the dark periods of the light:dark cycle. Values are hourly means % recording time ± SEM. Asterisks (*) indicate significant changes from HBSS. Infected mice had significant increases in NREM on days 7-11 after inoculation, significant decreases in REM and significant increases in transitions on days 7-9 after inoculation and significant decreases in waking on days 7-11 after infection ($p<0.05$).
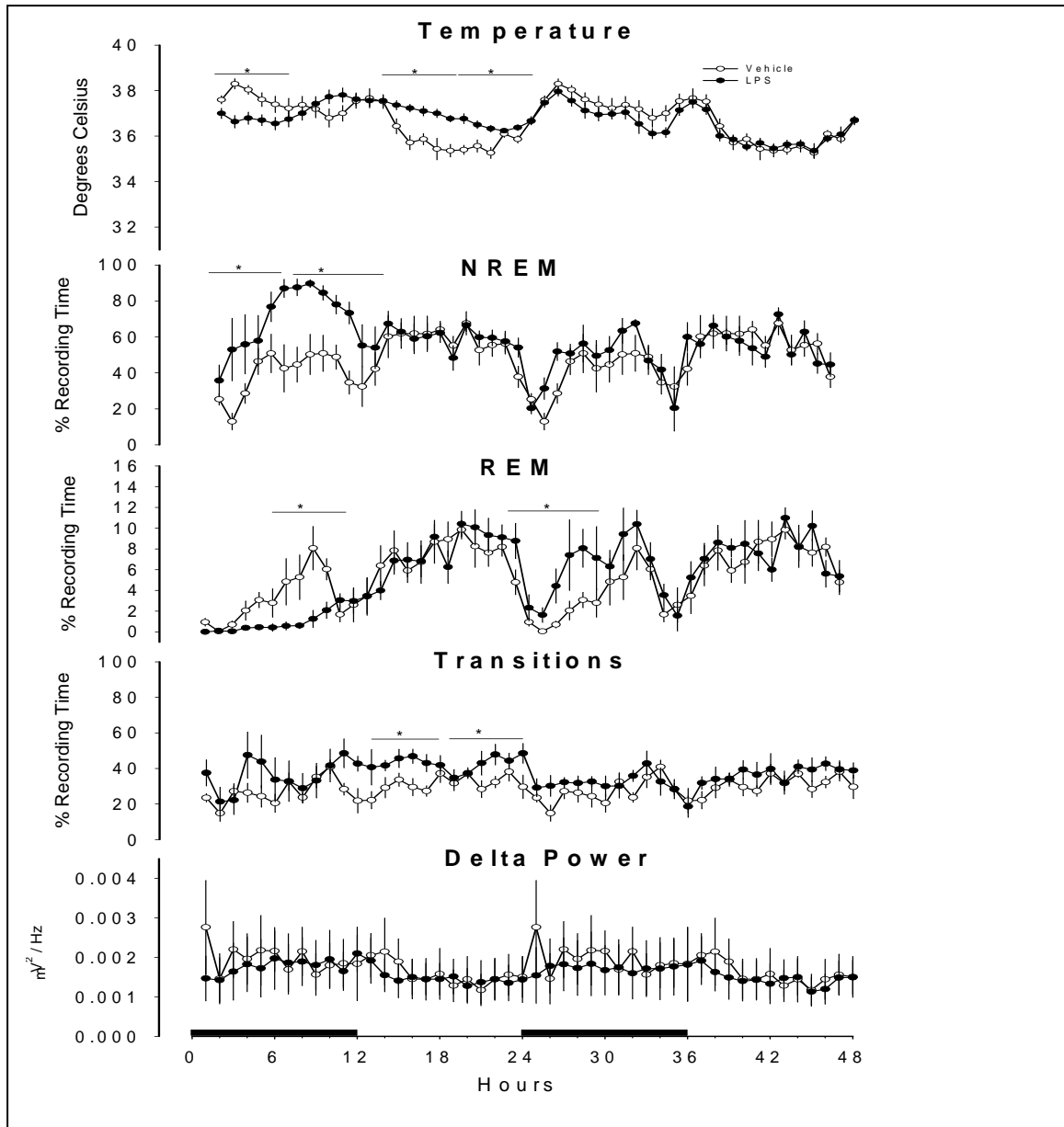
Figure 3.7: Impact of vehicle (n=6, open circles) and LPS (n=6 filled circles) on core body temperature, NREM sleep and REM sleep in uninfected C57BL/6J mice. Values are hourly means ± SEM. Dark bars signify dark periods of the light:dark cycle. Asterisks (*) signify differences from vehicle. LPS caused significant changes in temperature during hours 0-6 and 12-24, increases in NREM during hours 0-12 and decreases in REM during hours 7-12 and 24-30, as well as significant increases in transitions during hours 12-24 after injection.

Temperature

NREM

REM

Transitions

Delta Power

VEHICLE
LPS

Degrees Celsius

38.5
38.0
37.5
37.0
36.5
36.0
35.5
35.0
34.5

% Recording Time

100
80
60
40
20
0

% Recording Time

10
8
6
4
2
0

Transitions Per Hour

80
60
40
20
0

0.0040
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000

Hours

0   6   12   18   24   30   36   42   48   54   60

61
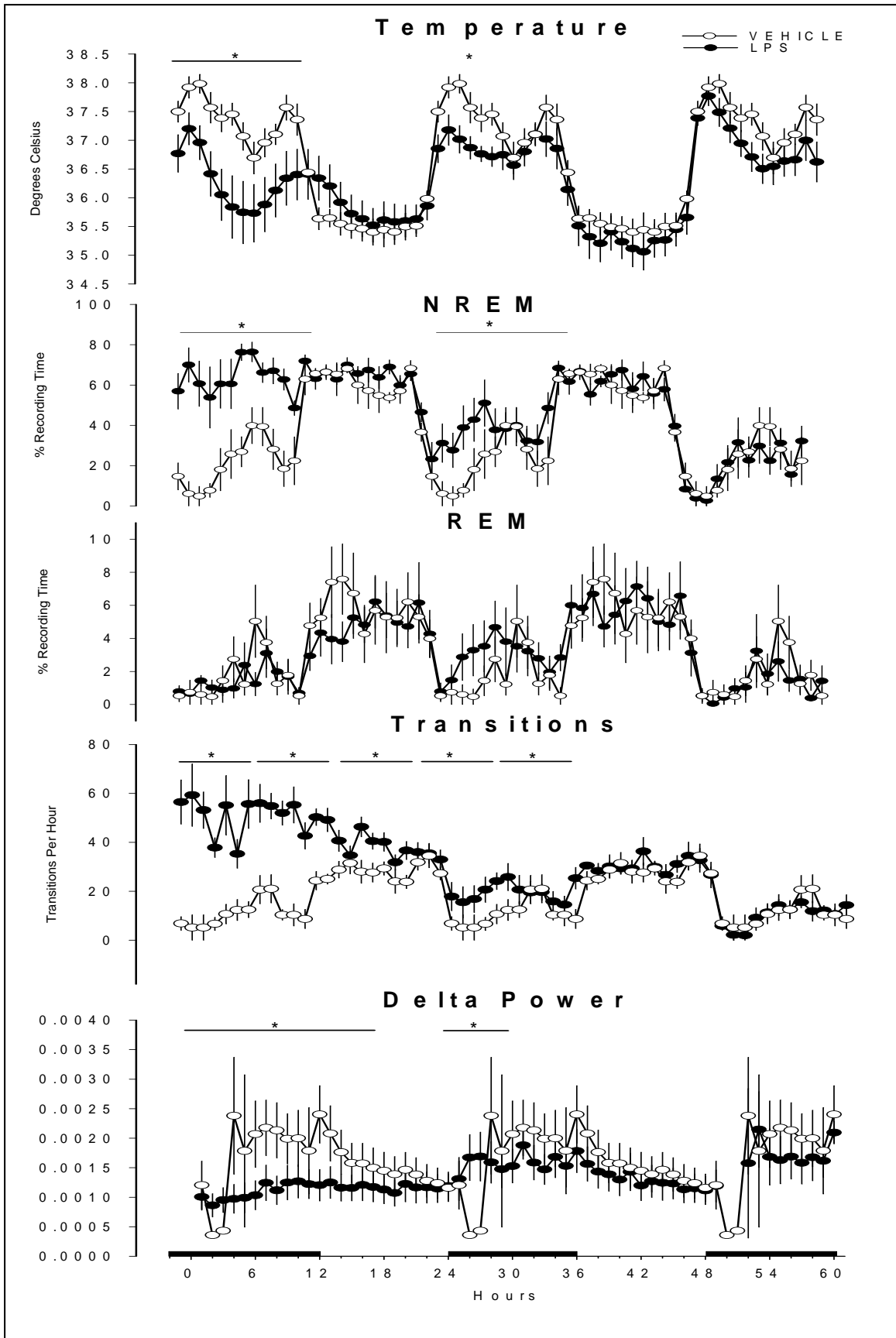
Figure 3.8: Impact of vehicle (n=13, open circles) and LPS (n=13, filled circles) in C57BL/6J mice infected with 400 PFU MuGHV on core body temperature, NREM, and REM sleep, transitions and delta power. Values are hourly means ± SEM. Dark bars signify dark periods of the light:dark cycle. Asterisks (*) indicate significant differences from vehicle. Infected mice had significant hypothermia during hours 0-12 and 24-30 after injection, as well as significant increases in NREM during hours 0-12 and 24-36, significant increases in transitions during hours 0-30 and significant decreases in delta power during NREM during hours 0-18 and 24-30 after injection with LPS compared to saline vehicle.

Temperature

Degrees C

NREM

% Recording Time

REM

% Recording Time

Transitions

Transitions per hour

Delta Power

mV^2 / Hz

Hours
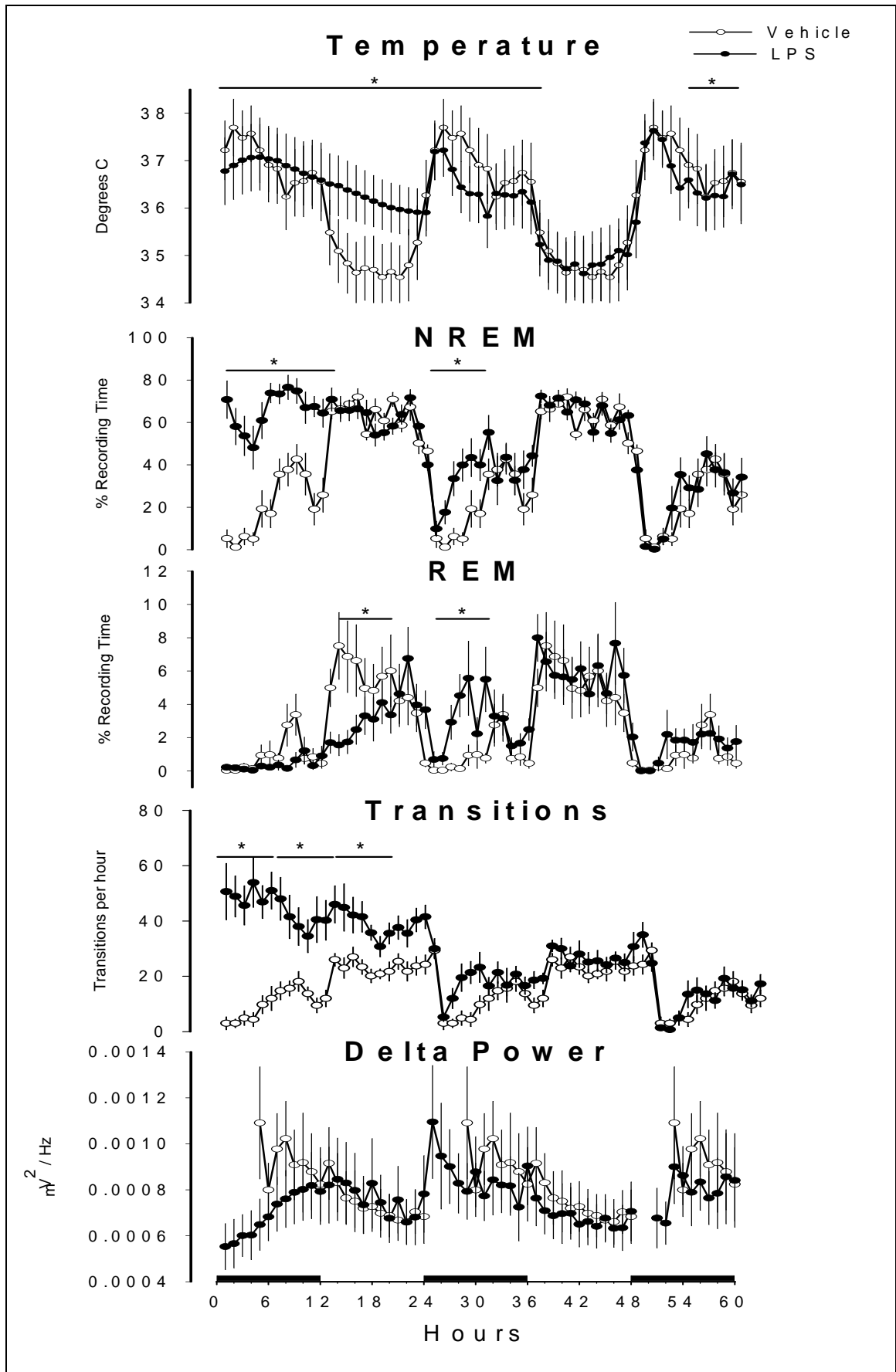
Vehicle
LPS

Figure 3.9: Impact of vehicle (n=8, open circles) and LPS (n=8, closed circles) in C57BL/6J mice infected with 40,000 PFU MuGHV on NREM and REM sleep, transitions, and delta power. Dark bars signify the dark portions of the light:dark cycle. Asterisks (*) signify differences from vehicle. Infected mice had significant changes in temperature during the first 36 hours after injection with LPS, as well significant increases in NREM during hours 0-12 and 24-30 after injection with LPS, significant decreases in REM during hours 12-18 and 24-30, significant increases in transitions during hours 0-24 after LPS.

Temperature

NREM

REM

Delta Power

Uninfected
400 PFU
40,000 PFU

Degrees Celsius

% Recording Time

mV^2 / Hz

Hours

65
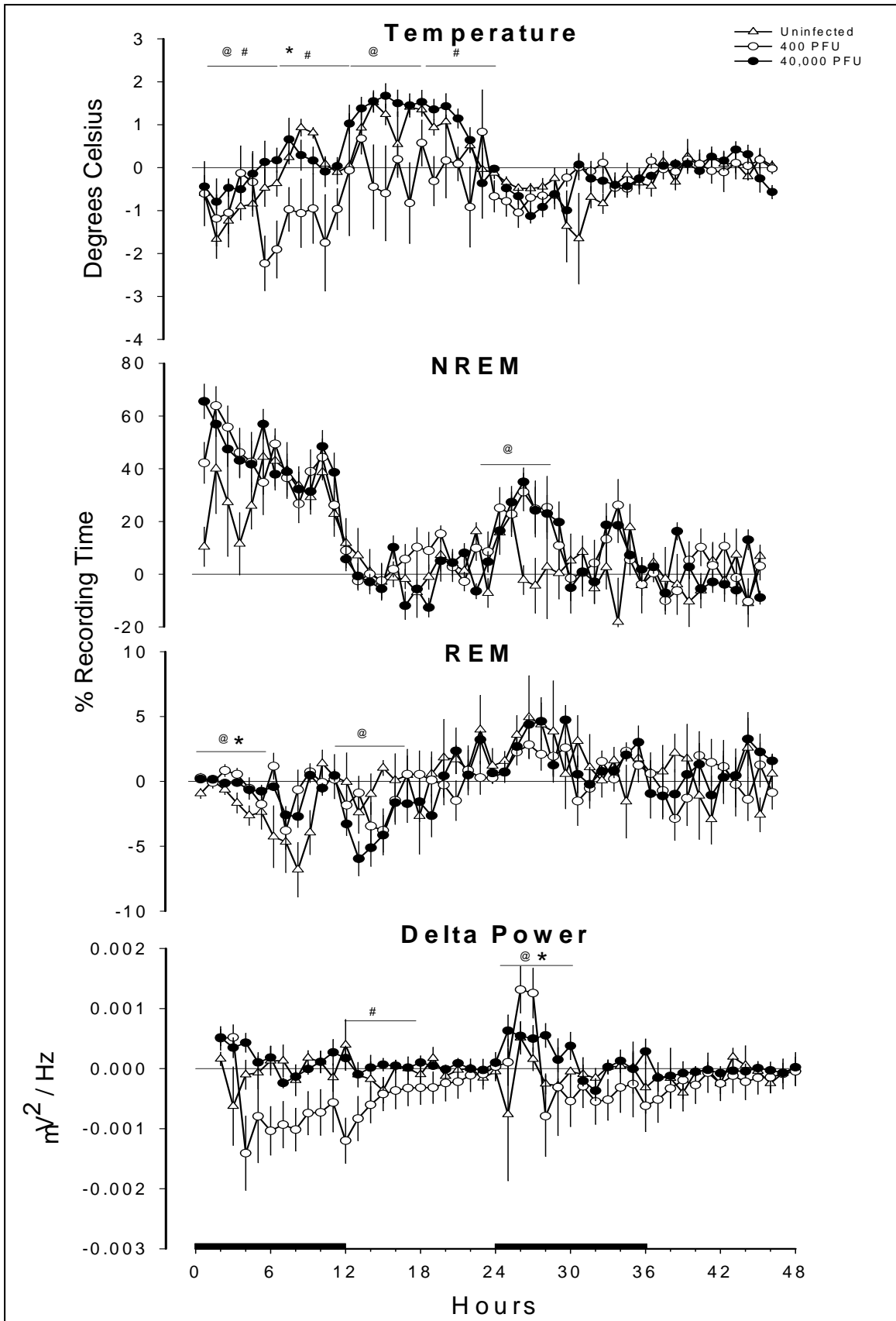
Figure 3.10: Differences in core body temperature, NREM sleep and REM sleep and NREM delta power between C57BL/6J uninfected mice (n=6, open triangles) and C57BL/6J mice infected with 400 PFU (n=13, open circles) or 40,000 PFU (n=8, closed circles) of MuGHV. Values are differences from respective vehicles (zero line) ± SEM. Asterisks (*) signify differences from 400 PFU infected and control mice, at (@) signs indicate differences between 40,000 infected mice and control mice, pound signs (#) indicate differences between 400 vs. 40,000 PFU infected mice. Significant differences in temperature were found between 40,000 PFU mice and uninfected mice during hours 0-6 and 13-18, and between both doses of infected mice at hours 7-12 and 19-24. 400 PFU infected mice significantly differed from uninfected mice at hours 7-12. Uninfected mice had significantly less changes in NREM during hours 24-30 after LPS and significantly different responses in REM during hours 0-6 and 13-18. Mice infected with 400 PFU had significantly less REM inhibition during hours 0-6 after LPS. Infected mice groups had significantly different delta power changes in response to LPS during hours 12-18, and both 40,000 PFU and 400 PFU infected mice had significantly different responses in delta power compared to uninfected during hours 24-30 after LPS.
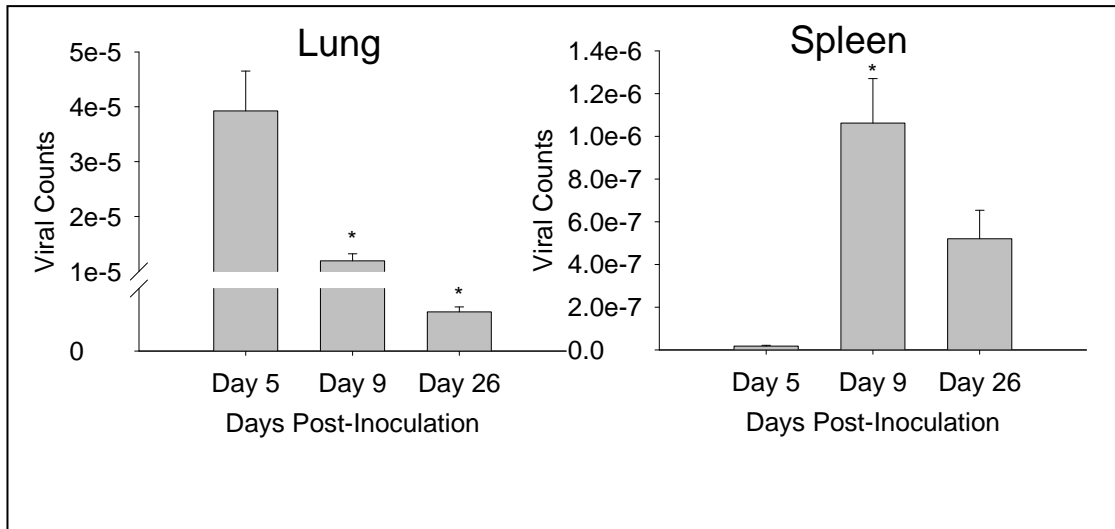
Figure 3.11. Copies of viral DNA in lung and spleen. Mice were sacrificed during active phase (day 5) and peak of lung infection (day 9) as well as during latency (day 26) after inoculation with 40,000 PFU of MuGHV. Virus was not detected in control mice (data not shown). Asterisks (*) indicate significant differences from day 5 (p<0.05).

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CHAPTER IV:

IMPACT OF MURINE GAMMAHERPESVIRUS INFECTION OF C57BL/6J MICE: CENTRAL AND PERIPHERAL CYTOKINE RESPONSES TO CHRONIC VIRAL INFECTION

Abstract

INTRODUCTION: Infection of mice with murine gammaherpesvirus-68 (MuGHV) is a model for human Epstein-Barr virus (EBV) infection. Sleep-wake behavior, activity patterns, and body temperatures of mice inoculated with MuGHV are altered in a manner that mimics some facets of nighttime sleep disruptions and daytime fatigue of individuals infected with EBV or symptomatic for chronic fatigue syndrome. MuGHV induces synthesis and secretion of the cytokines interleukin (IL)-1β and IL-6 in the spleen and lung. However, concentrations of cytokines in the central nervous system (CNS) during infection with this virus have not been widely investigated. Because IL-1β, IL-6 and other cytokines are implicated in the regulation of complex CNS processes, we hypothesize that alterations in sleep, activity and thermoregulation during chronic MuGHV infection may be mediated, in part, by actions of these cytokines. As a first step in testing this hypothesis we determined concentrations of cytokines in discrete brain regions and in peripheral organs and serum during infection of mice with MuGHV. We

also hypothesize that chronically infected mice will exhibit exacerbated cytokine responses to secondary immune challenge with lipopolysaccharide (LPS).

METHODS: Male C57BL/6J mice (n=6-8) were housed in microisolator cages under a 12:12 light:dark cycle at 29 ± 1ºC. Control mice were inoculated intranasally (i.n.) with vehicle (Hank's balanced salt solution, HBSS) and sacrificed after 4 days (n=16). Separate groups of mice were inoculated with 400 or 40,000 plaque forming units (PFU) MuGHV and sacrificed 5, 9, or 26 days later. Brain tissue (hypothalamus, hippocampus and cerebellum), blood, spleen, and lung were collected, snap frozen in liquid nitrogen, and stored for subsequent cytokine quantification.

RESULTS: Cytokine concentrations in brain and the periphery were altered by MuGHV. The pattern of cytokine responses in brain depended on the dose of MuGHV. Of interest, cytokine concentrations in brain and peripheral samples obtained from mice inoculated with 400 PFU of MuGHV changed in opposite directions early in the infection (day 5), with brain concentrations decreasing and concentrations in the periphery increasing. Secondary immune challenge with LPS increased IL-1β and IL-6 concentrations in CNS and peripheral structures of both infected and non-infected mice.

CONCLUSIONS: Despite an inability of MuGHV to invade the adult mouse CNS, central and peripheral cytokine concentrations were altered in infected mice. The observation that central and peripheral cytokine concentrations change in opposite directions early during the course of infection suggests actions of feedback mechanisms between the CNS and peripheral organs. Additional studies are needed to further assess mechanisms involved in post-viral fatigue of mice infected with MuGHV.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus (GHV) that establishes life-long latency in the host (Levitsky & Masucci, 2002). EBV and other GHVs have two distinct phases, an active infectious phase, and a dormant (latent) phase. During active infection, GHVs are transmitted from the mucous membranes into the lungs where there is viral replication, a proliferation of lymphocytes, and a release of pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-6 (Sarawar, Lee, & Giannoni, 2004; Sarawar et al., 1996; Jabs, Wagner, Schlenke, & Kirchner, 2000). After a variable length period of active infection, the virus then travels within infected B cells through the lymph and enters the spleen. When the virus enters the spleen it alters its own DNA into an episome that halts viral gene expression (Nash & Sunil-Chandra, 1994). The virus then becomes largely silent, dividing mainly with infected B-cells. Although the virus replicates only sporadically during latent infection, cytokine levels remain chronically elevated (Nicholas, 2005).

Numerous studies suggest EBV infection may be central to the pathogenesis of chronic fatigue syndrome (CFS) resulting from a secondary immune challenge that may cause permanent alterations in the immune system such as chronic cytokine release, and may perpetuate the state of chronic daytime fatigue (Glaser & Kiecolt-Glaser, 1998; Jones, 1988; Natelson et al., 1994). Hypotheses that EBV is involved in the etiology CFS derive mainly from observations that individuals with CFS have higher antibody titers to EBV (Glaser et al., 2005; Matthews, Lane, & Manu, 1991; Swanink et al., 1995). It is hypothesized that elevated circulating cytokines during EBV infection result in CFS and the post-viral symptoms of fatigue. Patients with CFS have higher IL-1 and IL-6 levels that coincide with periods of fatigue (Cannon et al., 1999), a relationship that does not

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exist in normal subjects or in CFS patients during exercise-induced fatigue (Gupta, Aggarwal, & Starr, 1999). Patients with CFS suffer other symptoms and often report poor quality nighttime sleep, characterized by insomnia, awakenings during the night, and unrefreshing sleep (Unger et al., 2004; Fukuda et al., 1994). EBV upregulates and sustains IL-6 production in human B-lymphocytes during infection (D'Addario, Ahmad, Morgan, & Menezes, 2000). IL-6 is implicated in several human sleep disorders that are characterized by excessive daytime sleepiness and fatigue, including insomnia (Burgos et al., 2006; Okun et al., 2004; Vgontzas, Papanicolaou, Bixler, & Chrousos, 1997; Vgontzas et al., 1999).

Many responses to peripheral immune challenge, such as alterations in sleep, body temperature, activity and food intake, are mediated by cytokine actions in brain (Dantzer, 2001; Dantzer et al., 1998; Dantzer et al., 1999). Pro-inflammatory cytokines such as IL-1β and TNFα regulate non-rapid eye movement (NREM) sleep (Opp, 2005; Krueger, Obal, Fang, Kubota, & Taishi, 2001) and are also principle mediators of the effects of GHV infection (Sarawar et al., 2004). These cytokines induce sickness behavior in animals and humans, as manifest by fatigue, fever, malaise, and arthralgia (Dantzer, 2001; Dantzer et al., 1998; Dantzer et al., 1999; Vollmer-Conna et al., 2004); all symptoms of CFS.

Although cytokines have been implicated as mediators of some symptoms of EBV infection and CFS, mechanistic studies in humans are difficult due to variability in the extent of infection, genetic background, immune status, and the subjective nature of fatigue ratings (Glaser, Padgett, 2005; Hossain, 2005). Murine GHV-68 (MuGHV) is widely regarded as a useful immunological model for EBV infection because it has

similar stages of active and latent infection, induces similar host-immune responses, has similar host immune evasion tactics, and shares genetic structure (Blackman & Flano, 2002; Flano, Woodland, & Blackman, 2002). MuGHV alters the secretion of cytokines implicated in the regulation of sleep, which may contribute to a state of chronic fatigue (Olivadoti, in preparation).

Whereas studies of MuGHV infection as an immunological model for EBV infection demonstrate similar patterns of cytokine release in the lung and spleen between EBV and MuGHV, no studies have determined the impact of MuGHV infection on concentrations of cytokines in the brain. Furthermore, it is not known how central and peripheral cytokine concentrations relate to behavioral changes during different stages of viral infection. In addition, many studies of EBV and MuGHV, have been conducted in vitro using infected cell lines (Sarawar et al., 1996). Although in vitro studies are useful in many respects, in vitro studies may not reflect concentrations of cytokines in freely-behaving animals during specific phases of MuGHV infection. We hypothesize that changes in CNS and/or peripheral cytokines will be associated with fatigue and alterations in behavior during infection with MuGHV and secondary immune challenge. We focus on brain regions that have well-established roles in sleep, fatigue, and thermoregulation, including the hypothalamus, brainstem, and hippocampus (Opp, 2005; Krueger et al., 2001), and on peripheral organs that are most likely to be effected by the virus. These peripheral organs include lung and spleen samples from infected and uninfected mice. We now report that infection of mice with MuGHV alters cytokine concentrations in brain and in the periphery.

Methods

<u>Animals</u>

Adult male (22-30g) C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were individually housed in microisolator cages and kept on a 12:12 hour light:dark cycle at an ambient temperature of $29 \pm 1°$ C. Water and rodent chow (Lab Diet 5001, PMI Nutrition International, Brentwood, MO) were provided <u>ad libitum</u>. All procedures were approved by the University of Michigan Committee on Care and Use of Animals in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and Use of Laboratory Animals.

<u>Viral Preparation and Inoculation Procedure</u>

Viral stock of MuGHV-68 (8.2×10^5 PFU/mL, Blackman Lab, Trudeau Institute) was stored at $-80°$ C and thawed just prior to use. To achieve a 400 PFU dose the stock was diluted to by adding 170 μL of virus to 10 mL of HBSS. 50uL of straight stock was used to inoculate mice in the 40,000 PFU dose group. Mice were lightly anesthetized with isoflurane and held upright by the scruff of the neck while 30 – 50 μl of fluid were placed into the nostrils (intranasal, IN) with a micropipette. Approximately half of the liquid was injected into each nostril and mice were observed until waking from the anesthesia.

<u>Protocols</u>

The same protocols used in previous studies to determine the impact of MuGHV

infection of mice on sleep-wake behavior, activity, and body temperature was used in this study (Figure 4.1). The time points at which animals were sacrificed in this study coincide with time points for which we collected data on behavior and thermoregulation. The animals used in this current study were housed in the same room under the same conditions as those used in previous studies conducted in our laboratory with this virus.

Experiment I: Impact of MuGHV infection on central and peripheral cytokine concentrations in C57BL/6J mice.

Mice were placed in microisolator cages containing a running wheel and allowed one week to habituate to the presence of the wheel. After the habituation period, mice were given either 30 μL or 50 μL of vehicle (Hank's balanced salt solution) i.n.. Four days later mice were inoculated IN with either 30 μL of vehicle containing 400 PFU of MuGHV or 50 μL of vehicle containing 40,000 PFU of MuGHV. Mice given vehicle (n=8) were sacrificed four days after IN administration. Mice given MuGHV were sacrificed at day 5 (n = 8), day 9 (n = 5), or day 26 (n = 8) after inoculation.

Experiment II: Impact of MuGHV infection on central and peripheral cytokine concentrations in response to secondary challenge in C57BL/6J mice.

Mice were inoculated IN with either vehicle, 400 PFU of MuGHV, or with 40,000 PFU of MuGHV. All mice were housed in their cages for 30 days post-inoculation. At dark onset of day 31 post-inoculation, mice were injected intraperitoneally (IP) with 2 mL of pyrogen-free saline (n = 8 / group) or with 10 μg of lipopolysaccharide (LPS, *Escherichia coli* serotype O111:B4, Sigma Aldrich, St. Louis, MO) in 2 mL of saline (n =

8 / group). Approximately 6 h after IP injection, mice were anesthetized and a blood sample was taken into a vacutainer tube containing EDTA (BD, Franklin Lakes, NJ). Mice were then euthanized and the lungs, spleen, and brain regions (brainstem, hypothalamus, hippocampus) were removed. All tissue samples were put in cryovials (VWR, West Chester, PA) and flash-frozen in liquid nitrogen before being stored at -80 ºC. Blood samples were spun for 20 minutes at 10,000 RPM at 4 ºC and plasma was removed and stored at -80 ºC.

Sample preparation and cytokine determination

Total protein was extracted from tissue samples (Hulse, Kunkler, Fedynyshyn, & Kraig, 2004) and concentrations determined using a BCA assay (Pierce, Rockford, IL). Protein amount was standardized to 200 μg for each sample. All serum samples were standardized by loading 50 μL into the plate. Cytokine concentrations were determined by using a multiplex assay (Bioplex; BioRad Laboratories, Hercules, CA) based on Luminex® suspension array technology. Concentrations of IL-1β, IL-6, IFNγ and TNFα, were determined for all samples. Lung and serum samples additionally were assayed for the chemokine RANTES (CCL5).

Statistical analyses

Statistical analyses were performed using SPSS v. 13.0 for Windows. Data were evaluated using unpaired T-tests for between and within dose concentrations. Comparisons were made between conditions (vehicle, MuGHV dose) within each structure or sample. An alpha level of 0.05 was accepted for all statistical tests as

indicating significant departures from control values. Percent change [(experimental value – control value)/ control value x 100] was calculated for visual representation of data.

Results

Infection of mice with MuGHV induced alterations in cytokine concentrations in brain, peripheral organs, and serum (Figure 4.2). There were differences in cytokine concentrations in samples obtained at different time points from mice inoculated with 400 PFU of MuGHV or 40,000 PFU of MuGHV. Overall, the pattern of change in cytokine concentrations during the course of infection with this virus reflects a dynamic process in which profiles of cytokines are altered.

Experiment I: Impact of MuGHV infection on central and peripheral cytokine concentrations in C57BL/6J mice.

Spleen

Splenic cytokine concentrations were altered during the course of infection with MuGHV (Figure 4.2). Concentrations of IL-1β and IFNγ increased ($p < 0.05$) in spleens obtained from mice inoculated with 400 PFU MuGHV and sacrificed on day 9 post-inoculation. IFNγ remained elevated ($p < 0.01$) in samples obtained on day 26 post-inoculation. Cytokine concentrations in spleens obtained from mice inoculated with 40,000 PFU MuGHV also were altered relative to samples obtained from control animals. IL-6 ($p < 0.01$) and IFNγ ($p < 0.05$) were elevated in samples obtained on days 9 and 26

post-inoculation, respectively. IL-1β concentrations decreased ($p < 0.01$) in spleens obtained on day 5 post-inoculation with 40,000 PFU MuGHV. Concentrations of IL-1β and IFNγ on day 9 after inoculation were proportionally higher ($p < 0.05$) in spleens obtained from mice inoculated with 400 PFU than from those obtained from mice inoculated with 40,000 PFU. By day 26 post-inoculation, concentrations of IFNγ were greater ($p < 0.05$) and TNFα were lower ($p < 0.001$) in spleens from mice inoculated with 40,000 PFU muGHV as compared to spleens from mice inoculated with 400 PFU.

Lung

Concentrations of cytokines in lung were also altered during the course of infection with MuGHV (Figure 4.5). Lung samples from mice infected with 400 PFU had significantly higher RANTES concentrations ($p=0.001$, Figure 4.3) on day 26 compared to lungs from control mice. Lungs from mice infected with 40,000 PFU of MuGHV had significantly higher concentrations of IFN-γ on day 5 after viral inoculation ($p<0.05$), as well as significant higher concentrations of RANTES on day 9 ($p<0.001$) and 26 ($p=0.001$) after inoculation compared to concentrations in lungs from control mice. Lungs from infected mice had significantly different concentrations of TNFα on day 26 ($p<0.05$), as well different concentrations of IFN-γ from lung samples taken on day 5 and 9 after infection ($p<0.05$).

Brainstem

Brainstem samples from mice inoculated with 40,000 PFU did not show significant differences in concentrations of cytokines compared to control mice.

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However brainstem samples from mice inoculated with 400 PFU had significant differences in cytokine concentrations compared to samples from vehicle treated mice. Samples collected 5 days after infection from mice inoculated with 400 PFU had significantly higher concentrations of TNF-α (p<0.05) compared to brainstem samples from vehicle treated mice. Samples collected from 400 PFU infected mice on day 9 had lower concentrations of IL-1β (p<0.05), IL-6 (p<0.01), IFN-γ (p=0.001), and TNF-α (p<0.001) than vehicle samples collected on day 9 after infection. When comparing changes in concentrations in brainstem samples taken on day 9 after infection, samples from mice inoculated with 400 PFU of MuGHV had significantly lower concentrations of IL-6 (p<0.001), IL-1 (p<0.010), IFN-γ (p<0.001) and TNF-α (p<0.05) compared to samples taken from 40,000 PFU infected mice.

Hippocampus

Cytokine concentrations in hippocampus samples obtained from infected mice were significantly different compared to samples from uninfected mice and when comparing concentrations between doses (Figure 4.2). Samples from mice infected with 40,000 PFU had higher concentrations of IL-1β on day 9 and 26 after infection (p<0.05) and concentrations of IL-6 in hippocampus were significantly higher on day 5 (p<0.001), 9 (p<0.05), and 26 (p=0.001) after inoculation with 40,000 PFU compared to hippocampus samples from uninfected mice. Hippocampus samples from the 40,000 PFU infected mice also had significantly higher concentrations of IFNγ on day 26 after inoculation compared to hippocampus samples of vehicle treated animals. Hippocampus samples taken from mice inoculated with 400 PFU MuGHV had significantly higher

concentrations of IFNγ on day 9 (p<0.05) after infection, and higher concentrations in TNFα on days 9 and lower concentrations on day 26 after infection compared to samples from uninfected mice (p<0.05). When comparing cytokine concentrations in hippocampus between doses of infected mice, samples from 40,000 PFU mice had significantly lower IL-6 and IL-1β concentrations collected on day 9 after infection (p<0.05), and significantly different values in TNF-α in samples collected on day 26 (p<0.05). In addition samples from 40,000 PFU mice had significantly different concentrations of IFN-γ on days 5, 9 and 26 after infection (p<0.05) compared to samples from mice infected with 400 PFU on the corresponding days of infection.

Hypothalamus

Hypothalamus samples taken from mice infected with 400 PFU had significantly lower concentrations of IL-1β collected on day 5 after inoculation (p<0.05) than control mice, as well as IL-1 (p<0.05), IL-6 (p<0.001), IFN-γ (p<0.05), and TNF-α (p<0.05) collected on day 9 after infection. Samples taken from mice infected with 40,000 PFU had significant decreases in IL-1b on day 5 after inoculation. Mice infected with 400 PFU had significantly lower IL-1 levels than samples taken from mice infected with 40,000 PFU taken on day 5 (p<0.01), as well as significantly lower IL-6 concentrations in samples taken on days 5 (p<0.01) and 9 (p<0.001) after inoculation. IFNγ responses differed significantly between doses on day 9 (p<0.05) and day 26 (p<0.05) after infection. In addition, TNF-α concentrations were significantly lower in samples from 400 PFU infected mice on days 9 and 26 compared to their samples from mice infected with 40,000 PFU (p<0.05).

<u>Serum</u>

Serum concentrations from infected mice differed from those of control mice. IL-1β and IL-6 concentrations were significantly higher in samples taken from 400 PFU on day 9 after infection and in samples from 40,000 PFU infected mice collected on day 5 after infection compared to samples from vehicle mice ($p<0.05$, Figure 4.2). IFNγ concentrations were significantly higher in samples taken on day 26 after inoculation with 400 PFU of MuGHV, and significantly increased in samples taken from mice inoculated with 40,000 PFU collected on days 5 and 9 after infection compared to vehicle mice. Samples taken from mice inoculated with 400 PFU of virus had significantly lower concentrations of RANTES (Figure 4.3) compared to serum from vehicle treated mice on day 5 after infection ($p=0.001$). Concentrations of IL-1β and IL-6 were significantly different in samples from mice inoculated with 40,000 PFU on days 5 and 9 after inoculation compared to samples from mice infected with 400 PFU. Samples from mice inoculated with 40,000 PFU MuGHV were also significantly different in concentrations of IFNγ and RANTES on day 5 after inoculation and in TNF in samples collected on day 9 compared to samples from mice inoculated with 400 PFU MuGHV ($p<0.05$).

<u>Experiment II: Impact of chronic MuGHV infection in C57BL/6J mice on central and peripheral cytokine concentrations in response to secondary challenge</u>

<u>Spleen</u>

Concentrations of IL-1β were significantly increased in spleen samples from

uninfected mice infected mice 6 h after LPS compared to samples from their respective vehicle control groups (p<0.001, Figure 4.4). Spleen samples from mice infected with 400 PFU also had significant increases in concentrations of IFN-γ (p<0.01) and IL-6 (p=0.001, Figure 4.4) compared to 400 PFU inoculated and vehicle control mice. Spleen samples from both doses of infected mice had significantly different concentrations in TNF-α compared to samples from uninfected mice in the response to LPS (p<0.01). In addition, samples from mice inoculated with 400 PFU of MuGHV had significantly higher concentrations of IFN-γ (p<0.05) compared to samples from uninfected mice. There were no significant differences in spleen cytokine concentrations when comparing samples from two dose groups to each other.

Lung

Lung samples from uninfected mice and mice inoculated with 400 PFU had significantly higher concentrations of RANTES after LPS compared to lung samples from their respective vehicle controls (Figure 4.5, p<0.015). However, only samples from inoculated mice had significant increases in IL-1β concentrations in response to LPS compared to samples from the respective control groups (p<0.05 Figure 4.4). Lung samples from mice inoculated with 400 PFU had significantly different cytokine concentrations of IL-6 compared to samples from uninfected mice (p<0.05).

Brainstem

Brainstem samples from uninfected mice and mice inoculated with both doses had significantly higher cytokine concentrations of IL-6 in response to LPS compared to

samples from the respective vehicle control groups (p<0.010 all groups). Brainstem samples from uninfected mice and mice inoculated with 400 PFU also had significantly higher concentrations of IL-6 in response to LPS (p<0.05). Brainstem samples collected from mice inoculated with 40,000 PFU were significantly higher in concentrations of IL-1β and IL-6 compared to uninfected LPS treated mice samples (p<0.05). Samples from mice inoculated with 400 PFU had higher concentrations of IL-6 in response to LPS compared to samples from mice inoculated with 40,000 PFU of virus (p<0.02).

Hippocampus

Infected mice did not demonstrate the same LPS-induced changes seen in uninfected mice in Hippocampal cytokine concentrations. Hippocampus samples from uninfected control mice had significantly higher concentrations of IL-1 and IL-6 in response to LPS compared to sample concentrations from uninfected vehicle mice (p<0.010, Figure 4.4). Samples from uninfected mice had significantly different cytokine concentration patterns of IL-1 (p<0.05) and IL-6 (p<0.010) responses to LPS compared to samples collected from mice inoculated with 40,000 PFU.

Hypothalamus

Hypothalamus samples collected from uninfected mice and mice inoculated with 400 PFU had significantly higher concentrations of IL-6 (p<0.05, Figure 4.4) in response to LPS compared to samples from their respective control groups. Samples from uninfected mice had significantly less alterations in concentrations of IL-1 and IL-6 in response to LPS compared to samples from mice inoculated with 400 PFU (p<0.05). In

addition, hypothalamus samples from uninfected mice had significantly different TNF-α concentration response pattern when compared to samples from 40,000 PFU inoculated mice (p<0.010). Samples from mice infected with 400 PFU had significantly larger TNF concentration increases in response to LPS compared to samples from 40,000 PFU inoculated mice (p<0.01).

Serum

Serum samples collected from uninfected mice had significant changes in concentrations of IL-1β, IL-6, IFNγ, and TNF-α in response to LPS compared to vehicle mice (p<0.05, Figure 4.4). Samples collected from mice infected with 400 PFU MuGHV had significant changes in IL-1β, IL-6, and IFNγ in response to LPS compared to samples from infected vehicle mice. Samples from mice inoculated with 40,000 PFU had significant changes in IL-1β compared to samples from infected vehicle mice. In addition, samples from both uninfected and infected mice had significant changes in concentrations of RANTES in response to LPS compared to their respective vehicle groups (p<0.05, Figure 4.5). Comparing cytokine concentrations in response to LPS, samples from non-infected mice had significantly different changes in IL-6, IFN, and TNF-α compared to samples from mice infected with 400 PFU of MuGHV (Figure 4.4 p<0.05) and RANTES (Figure 4.5, p<0.05). Samples from uninfected mice had significantly different changes in concentrations of TNF-α in response to LPS compared to samples from mice inoculated with 40,000 PFU MuGHV. Concentrations of RANTES in serum samples in response to LPS significantly differed between different dose groups (Figure 4.5, p<0.05).

Discussion

Infected mice have different response patterns in cytokine concentrations at different points of infection depending on the dose of infection, indicating a dose-specific response that is less clearly defined than previously suggested. However an interesting caveat in these findings is that cytokines that are normally pyrogenic and fatigue-inducing, including IL-1β, IL-6 and IFNγ are elevated in peripheral organs, yet suppressed in CNS tissues at times of chronic hypothermia and fatigue, suggesting interactions between central and peripheral systems to modulate complex behaviors in response to viral infection. Significant increases in IL-6 on day 9 after infection of 40,000 PFU could explain the significant decreases in food intake, body weight and activity. However increases in RANTES in mice inoculated with 40,000 PFU MuGHV, which should normally cause fever (Tavares, et al., 2000), occurs at a time of pronounced hypothermia in these mice. These differences in cytokine changes in response to infection and secondary immune challenge may explain differences in dose-related behavioral responses to infection. Yet pro-inflammatory and pyrogenic cytokines, such as IL-1β and IL-6 are high in both uninfected and viral-inoculated animals in response to LPS, yet create different resulting body temperature and sleep profiles. These results suggest that there is more at play in this virus causing changes in behavior than simply cytokine interaction, and cytokine concentrations in samples from freely-behaving animals are much more dynamic than in vitro samples.

Patterns of sleep-wake behavior, activity, and body temperature of mice infected with MuGHV are altered in a manner that mimics some facets of nighttime sleep

disruptions and daytime fatigue of individuals infected with EBV or symptomatic for chronic fatigue syndrome (Olivadoti, et al., 2007; Chapter 3). The peak of active infection in lungs of mice inoculated with MuGHV occurs between days 7 – 14, after which the virus becomes latent in the spleen (Flano, et al., 2002). Recent behavioral data demonstrate that the time course of infection with 40,000 PFU of MuGHV, in particular the peak of active infection in the lung, is associated with significant changes in wheel running activity, core body temperature, food intake and body weight (Olivadoti, Chapter 3). Wheel running of mice infected with 400 PFU of MuGHV or with 40,000 PFU of MuGHV progressively decreases during infection and there are chronic persistent changes in core body temperature (Olivadoti, in preparation). Mice infected with MuGHV also have altered responses to secondary immune challenge with LPS, including abnormal patterns of body temperature and alterations in sleep-wake behavior (Olivadoti, Chapter 3).

Pro-inflammatory cytokines, such as IL-1β, TNF-α, IFNγ and IL-6 induce sickness behavior, as manifest by fatigue, fever, malaise, and arthralgia (Dantzer, 2001; Dantzer et al., 1998; Dantzer et al., 1999; Vollmer-Conna et al., 2004). In addition they also regulate changes in sleep in response to pathogens, such as fragmentation of sleep, increases in NREM and decreases in REM sleep (Krueger, Toth, Opp, Kimura-Takeuchi, & Kapás, 1991, Opp, 2005).

Infection with MuGHV alters cytokine synthesis and secretion in a manner that varies with the stage of infection. The active phase of MuGHV infection is associated with splenic production of IL-1β, IL-2, IL-6, IL-10 and IFN-γ (Sarawar et al., 1996). IFNγ is vital for clearance of virus from the lung via the induction of cytotoxic T cells,

natural killer cells, and macrophages and controls the latent phase by curtailing viral reactivation from latency (Dutia, Clarke, Allen, & Nash, 1997, Sarawar et al., 1997). IL-6 is present in elevated concentrations in blood during active MuGHV infection (Sarawar et al., 1996). IL-10 also influences MuGHV pathogenesis. For example, IL-10 knockout mice have reduced viral load in the spleen yet increased splenomegaly (Peacock, et al., 2001). During latent infection of mice with MuGHV, IL-1β, TNF-α and IL-6 concentrations are elevated (Sarawar et al., 1996). In addition, MuGHV infection induces high levels of RANTES in the lung throughout infection (Weinberg, Lutzke, Alfinito, & Rochford, 2004).

Whereas many studies have focused on peripheral cytokines as mediators of behavioral responses to infection, our results suggest that central mechanisms may also be important in regulating behavioral responses to MuGHV. Although this virus does not infect the central nervous system when given i.n. (Terry, Stewart, Nash, & Fazakerley, 2000), central structures do respond to infection with MuGHV as evidenced by changes in brain-regulated behavior and physiological processes during infection (Olivadoti, Chapter 3). For example, during the peak time frame of active infection, inoculated mice begin to show significant chronic fatigue and decreases in body weight and food intake, which coincides with increases in IL-1β and IL-6 in serum. However, cytokine concentrations do not follow chronic patterns along with behavioral changes. In addition, in mice inoculated with 400 PFU of MuGHV, concentrations of cytokines in brain decrease while cytokine concentration in peripheral samples increase. It is possible that infected mice may have developed a low-level infection in the CNS that has not been detected by previous studies. However it is more plausible that the changes in opposite

directions of central and peripheral concentrations of cytokines and the dynamic changes of cytokine concentrations despite chronic behavioral changes suggest possible negative feedback inhibitory mechanisms, such interaction with the hypothalamic pituitary adrenal (HPA) axis, are involved in the complex behavioral and physiological processes induced by this virus.

The HPA axis and immune system, particularly cytokines, work together through negative feedback mechanisms during infection to stop the ill-effects of chronic immune activation. Many studies have shown that cytokines such as IL-1β, IL6, TNFα, IFN-γ can alter the axis at all levels—from the release of corticotrophin releasing hormone (CRH) to adrenocorticotropin hormone (ACTH) in the brain, and finally to cortisol release from the adrenals, causing inhibition of these same cytokines at all levels depending on where concentrations are highest [reviewed in (Silverman, Pearce, Biron, & Miller, 2005)]. Thus it is plausible that the HPA axis is the moderator between peripheral and central cytokines, resulting in central inhibition during times of heightened peripheral release due to the peripheral nature of the virus.

In sum, our data demonstrate changes in both central and peripheral cytokines in response to infection with MuGHV. The dynamic relationship between central and peripheral cytokines and their impact on behavior are largely unclear, as many cytokines can have opposite effects of each other and play many different roles in physiology. Further studies are needed to understand the impact of physiological and immune changes in viral infection on behavior, including other mediators, as well as arousal and thermoregulatory mechanisms.
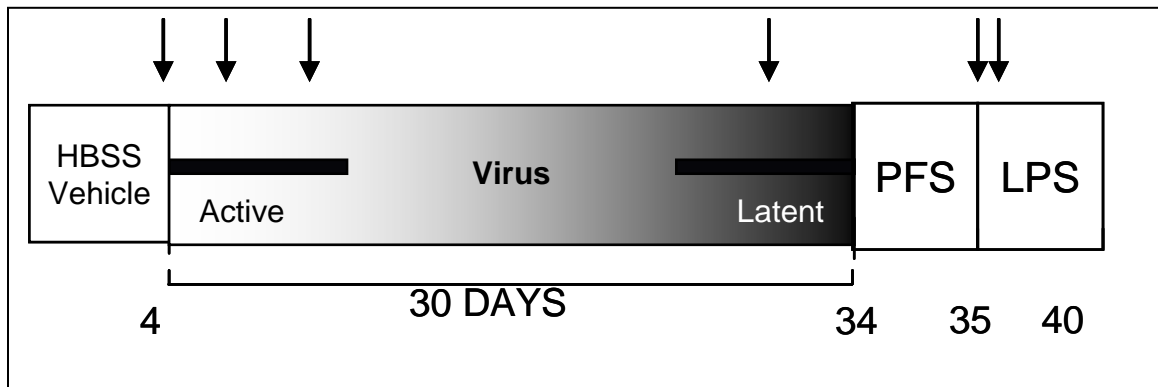
Figure 4.1. Graphic representation of protocol used to determine cytokine concentrations in brain and peripheral samples obtained from mice inoculated with MuGHV. Arrows indicate time points of the study at which mice were sacrificed. Control mice were sacrificed 4 days after vehicle administration (HBSS), 5-, 9- or 26 days after inoculation with MuGHV. In addition, separate groups of chronically-infected mice were sacrificed 24 h after intraperitoneal injection of pyrogen-free saline (vehicle), or 6 hours after intraperitoneal injection of 10 µg bacterial lipopolysaccharide injection.
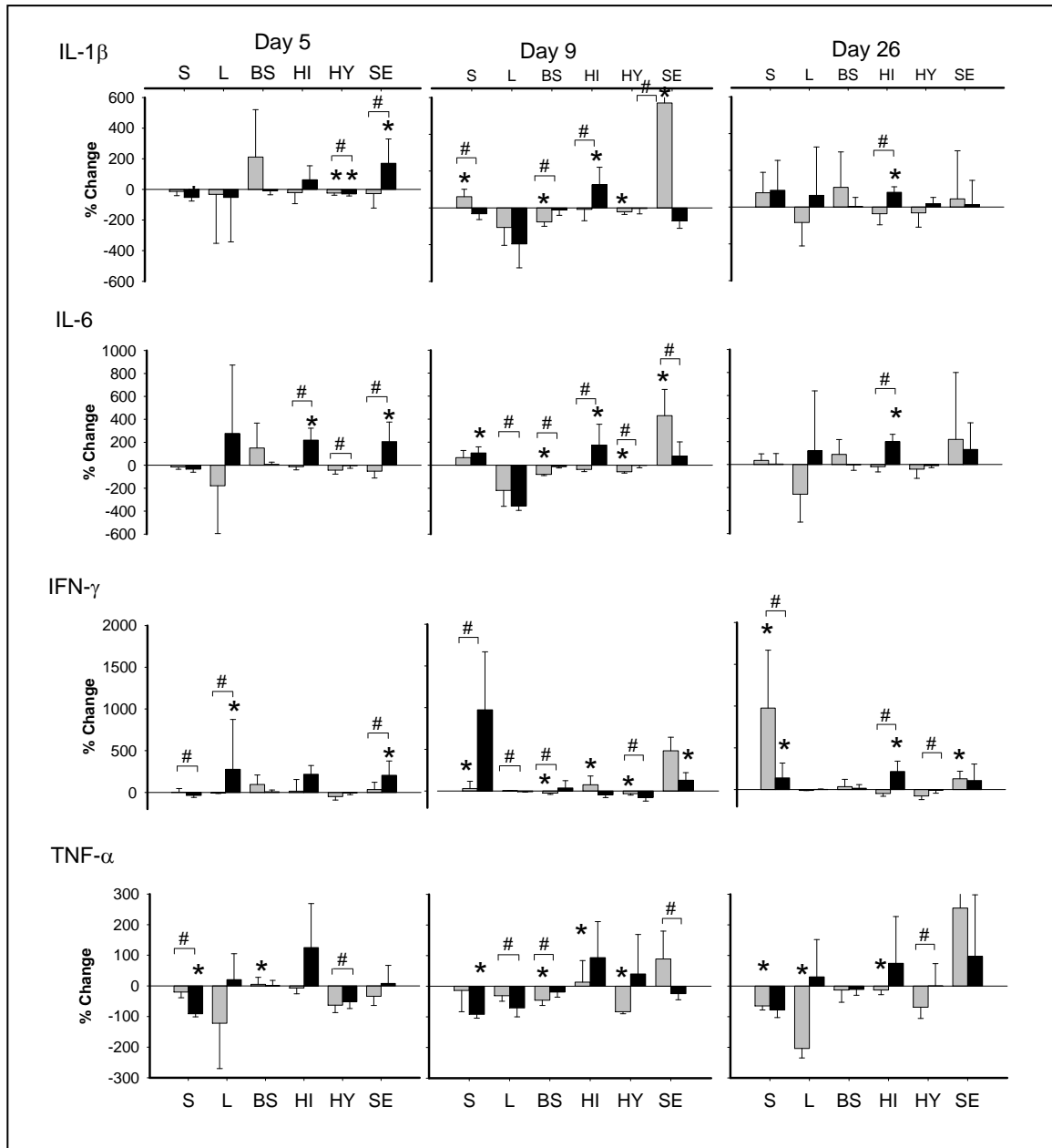
Figure 4.2: Impact of MuGHV infection of C57BL/6J mice on concentrations of IL-1β, IL-6, IFNγ and TNFα in spleen (S), lung (L), hippocampus (HI), hypothalamus (HY), and serum (SE) in mice infected with 400 PFU (grey bars, n=6-8) or 40,000 PFU (black bars, n=6-8) MuGHV. Values are expressed as percent change (± SD) relative to samples obtained from control animals. Asterisks (*) indicate significant differences from vehicle concentrations, pound signs (#) indicate differences between doses.
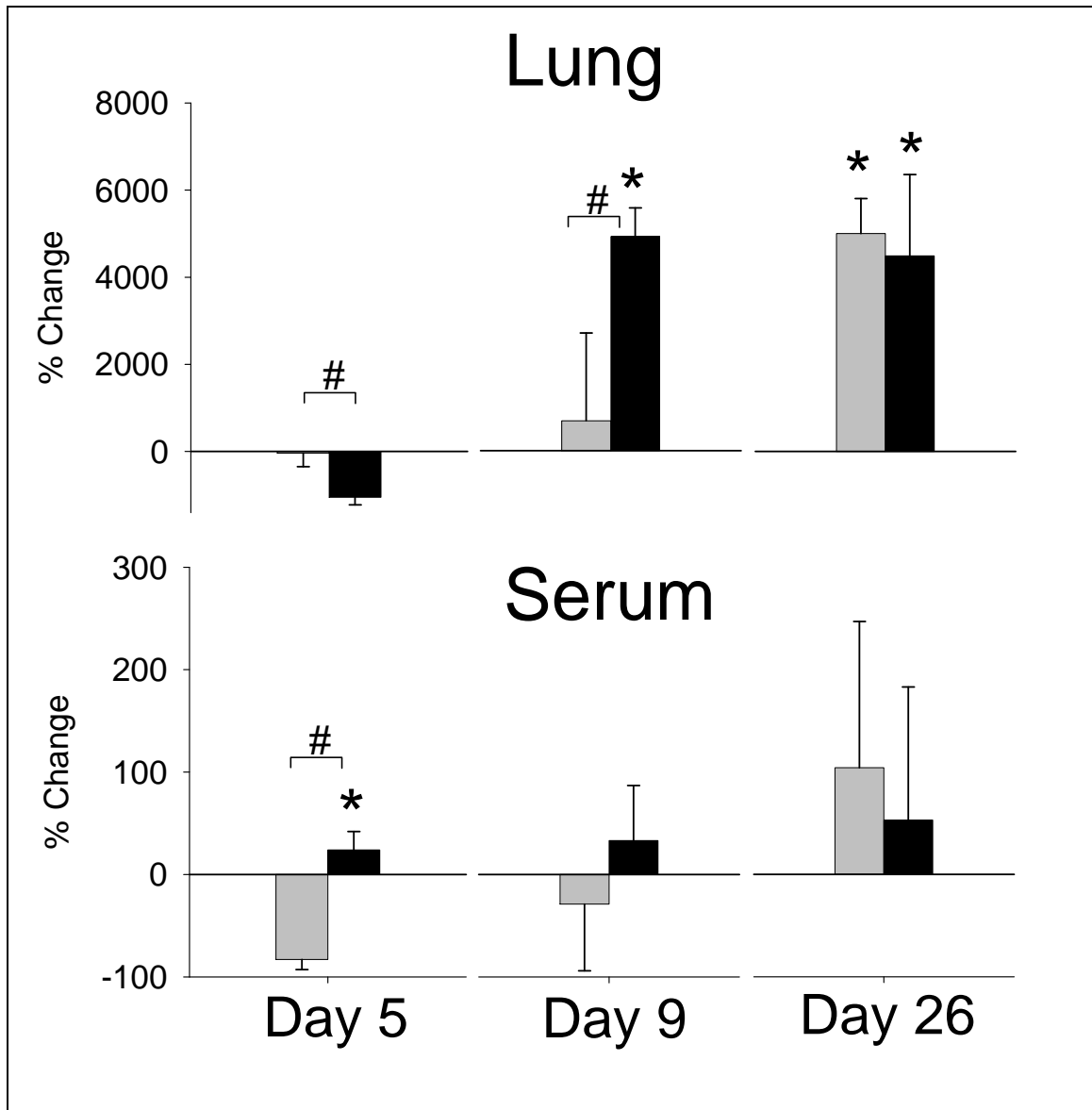
Figure 4.3: Impact inoculation of C57BL/6J mice with 400 PFU (grey bars) or 40,000 PFU (black bars) of MuGHV on concentrations of RANTES in lung and serum. Values are expressed as percent change (± SD) from values from samples obtained from control animals. Asterisks (*) indicate significant differences from vehicle concentrations, pound signs (#) indicate differences between doses.
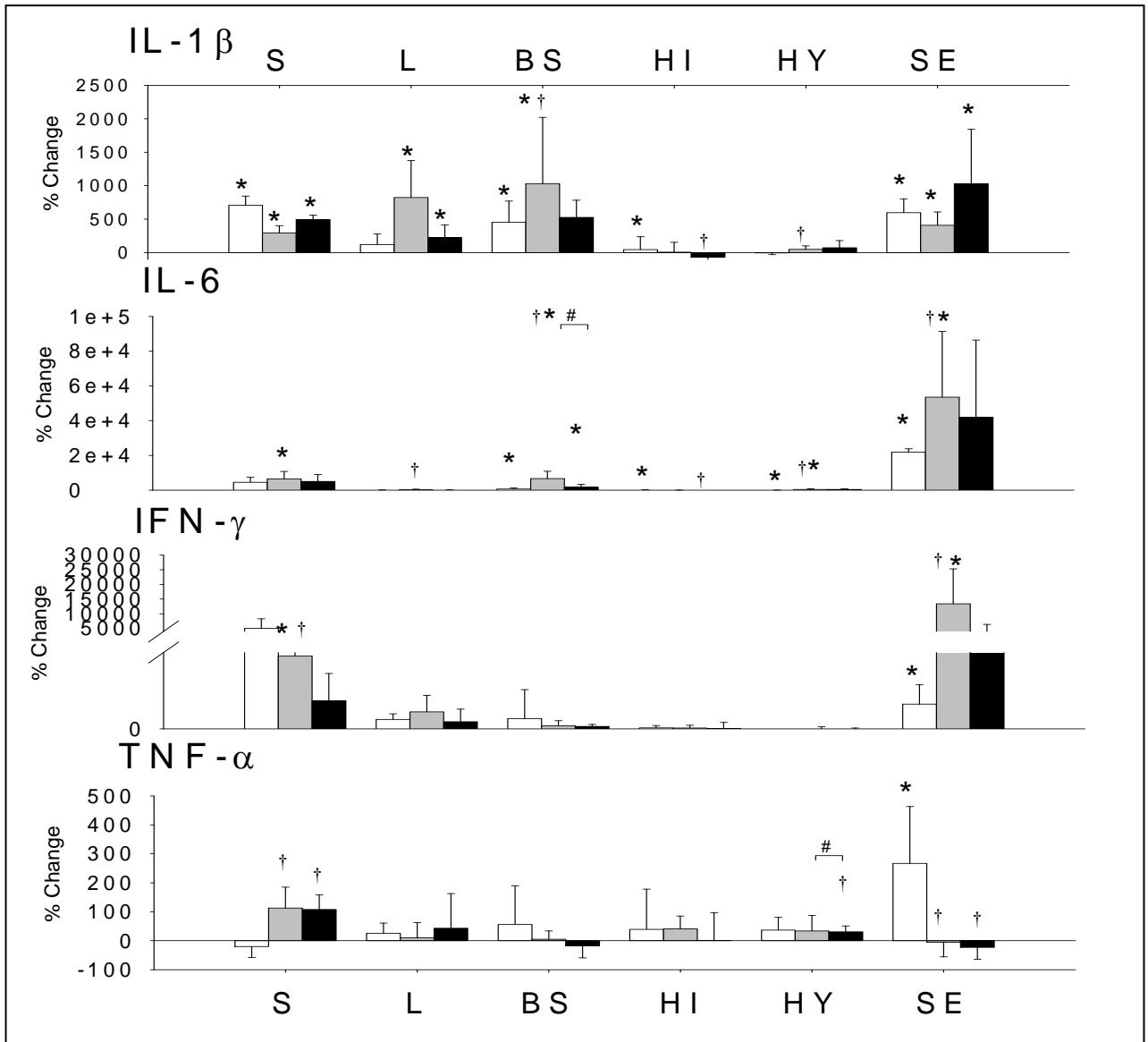
Figure 4.4: Impact of intraperitoneal injection of 10 μg lipopolysaccharide on IL-1, IL-6, IFNγ and TNFα concentrations in uninfected mice (white bars), mice inoculated with 400 PFU of MuGHV (grey bars), or mice inoculated with 40,000 PFU of MuGHV (black bars). Values are presented as percent change (± SD) from concentrations detected in samples obtained from control animals. S: spleen; L: lung; HI: hippocampus: HY: hypothalamus; SE: serum. Asterisks (*) indicate significant differences from vehicle, pound signs (#) signify differences between doses, cross signs (†) indicate difference from uninfected mice.
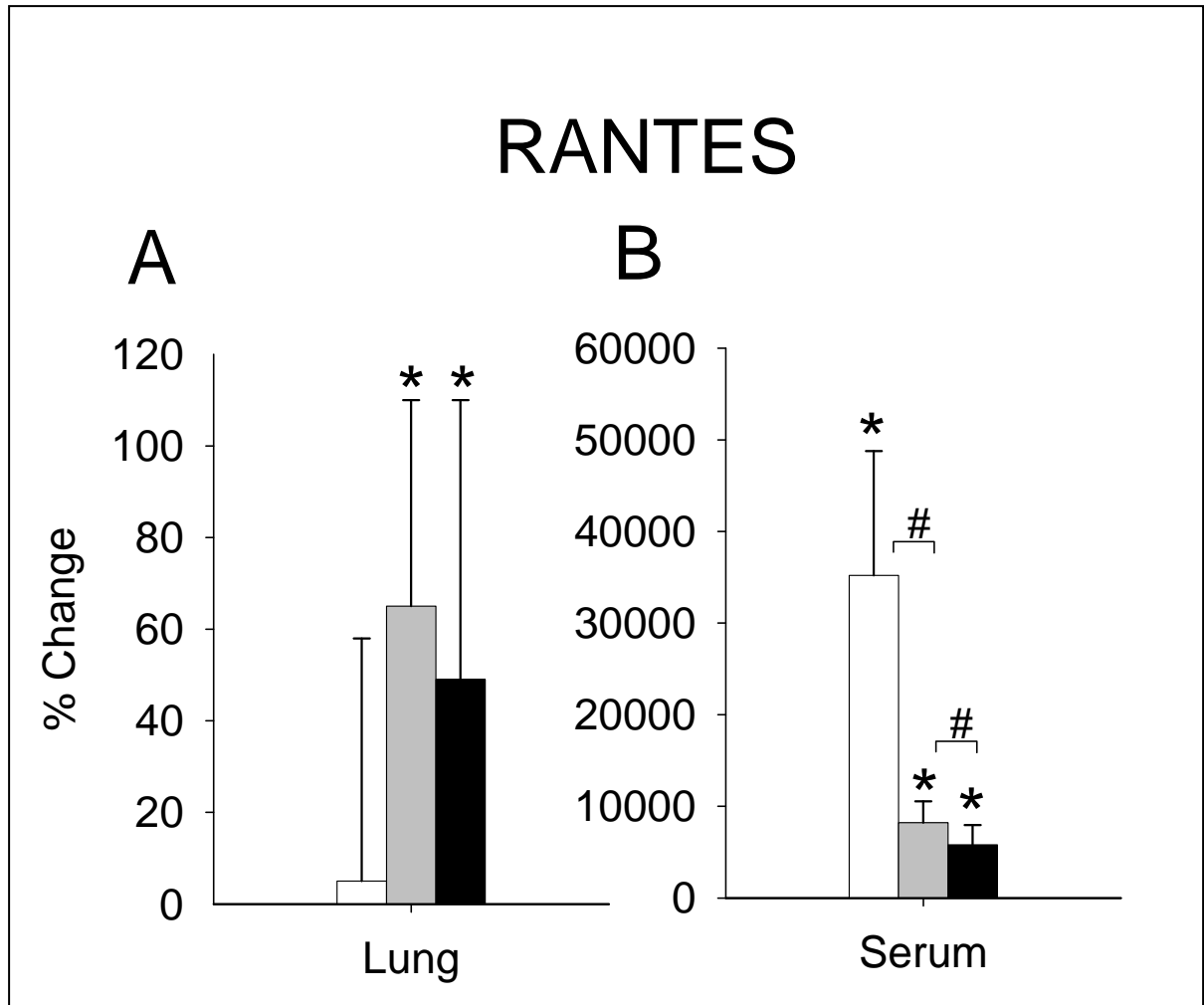
Figure 4.5: Impact of 10 μg lipopolysaccharide on RANTES concentrations in lungs obtained from uninfected mice (white bars), mice inoculated with 400 PFU of MuGHV (grey bars), or mice inoculated with 40,000 PFU MuGHV (black bars) in lung (A) and serum (B). Values are presented as percent change (± SD) from those obtained from samples of lung from control animals. Asterisks (*) indicate significant differences from vehicle, pound signs (#) signify differences between doses.

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CHAPTER V:

GENERAL DISCUSSION

Implications of MuGHV as a model for Epstein-Barr virus infection

Many people in the world that have been exposed to EBV are at risk for chronic fatigue due to this virus, as well as resulting conditions such as CFS and depression (Rickinson et al., 2001; Mandell et al., 1995). However not everyone who becomes infected with EBV ends up with chronic fatigue or CFS. Due to the difficulty of studying EBV and chronic post-viral fatigue, animal models are needed to study these two conditions in a controlled setting. Past research has demonstrated the immunological efficacy of using MuGHV as a model for human EBV (Blackman & Flano, 2002; Flano et al., 2002), and now our results have expanded their model to examine issues of post-viral behavior and fatigue to model EBV and resulting behaviors. Our results show behavioral correlates with infection, such as fatigue, changes in body temperature, and sleep, as well as abnormal responses to secondary immune challenge. These results mimic the human condition of fatigue and the severity of these changes increase according to inoculation dose. Thus these results further the notion that MuGHV is a well-adapted model for studying EBV and the pathogenesis of resulting chronic fatigue that occurs in some infected humans.

Compartmentalization of the virus and behavioral correlates

PCR results suggest that current time frames of infection in specific compartments of the body (i.e. lung vs. spleen) and resulting behaviors may be dose-specific. Thus more research on dosages of viral infection and the resulting behaviors may lead us to a better understanding of the interaction between immune responses to GHV infection and subsequent behavioral changes. Mice infected with MuGHV have sleep, temperature and body weight changes that are specific to the peak of viral infection of the active phase; however changes in these behaviors become more generalized during the latent phase.

Modeling of chronic post-viral fatigue

The resulting fatigue due to infection in our mice only occurs in voluntary wheel-running activity, while facultative activity, such as grooming, feeding, drinking, and nesting remains unchanged. This is an important facet of post-viral fatigue, as the definition of fatigue for CFS is a reduction in daily voluntary activity (Fukuda et al., 1994). Many CFS patients are able to maintain productivity in their work life, but find that it is difficult to maintain social relationships and an active family life (Rakib et al., 2005). However, like all syndromes, symptoms vary depending on the patient. In our model, mice infected with different doses of MuGHV have different degrees of fatigue, which suggests that the amount of virus that patients receive could affect their resulting fatigue. However further studies are needed to understand what other factors affect the resulting fatigue, including genetics, stress, and status of the immune system.

Thermoregulatory changes in mice infected with MuGHV

Infected mice have distinct changes in thermoregulation during the different stages of viral infection that vary by dose. However the paramount similarity is a hypothermia that is persistent into latency, suggesting changes in central thermoregulatory mechanisms, which also occurs in humans with CFS. However in CFS patients report chronic low-grade fevers, which raises the question of the physiological role of hypothermia in mice compared to fevers in humans. In addition, studies in adolescents with CFS have suggested abnormalities in catecholaminergic-dependant thermoregulatory mechanisms (Wyller et al., 2007) but the pathogenesis of these differences are still unknown. The role and mechanisms of hypothermia in mice are well-debated and still yet unclear, and thus makes it difficult for us to apply our results to humans with post-viral fatigue. More studies are needed to compare chronic hypothermia in MuGHV infected mice and chronic low-grade fevers in patients with post-viral fatigue.

Sleep Correlates

While mice infected with a high dose of MuGHV showed significant changes in sleep after infection that are similar to what is reported in EBV, these changes were limited to the acute phase of infection in the lung. However mice also lacked insomnia or fragmentation of sleep that could explain chronic fatigue, which makes MuGHV-induced changes a more pure fatigue state. It is unclear what generates chronic sleep problems in post-viral fatigue. Perhaps the sleep complaints in chronic fatigue patients are a factor of co-morbid conditions such as depression or other conditions such as fibromyalgia or

arthritis. Future studies would be helpful in understanding which changes cause chronic sleep variations in these patients.

Cytokine changes during infection

Our results replicated results of previous studies with increases in IL-1 and IL-6 in the lung and spleen throughout infection. However our results from central cytokines painted a much more complicated picture, suggesting both peripheral and central mechanisms at play during infection despite lack of viral invasion in the brain found in previous studies using adult mice (Terry, Stewart, Nash, & Fazakerley, 2000). Increases in peripheral cytokines (in the spleen and lung) occurred during simultaneous decreases in central cytokines (in the brainstem, hippocampus and hypothalamus) suggesting feedback mechanisms that must be able to communicate across the blood-brain-barrier. In addition, cytokine profiles across infection were different when comparing two different doses, suggesting that there is no clear general answer to how to address cytokine dysregulation in MuGHV infection. With the addition of complicated central cytokine profiles, there is no clear way to explain the thermoregulation changes, fatigue, and sleep disturbances in mice infected with MuGHV. For instance, there may be many different cytokines at play controlling hypothermia in 40,000 PFU mice, due to the large decreases in core body temperature at peak of infection in the lung, and subsequent chronic (but less severe) hypothermia after clearance from the lung. In addition, many cytokines can be both pyrogenic and cryogenic, or have a bi-phasic effect depending on the challenge, room temperature, and mouse strain used [reviewed in (Romanovsky et al., 2005)]. Thus it is prohibitive to draw conclusions about the role of cytokines in behavioral changes in MuGHV infection. Cytokine inhibitors and knock-out studies

would be helpful in understanding the role of central and peripheral cytokines in changes in behaviors during and after infection.

Abnormal Responses to Secondary Immune Challenge

Our results show dose-dependant changes in body temperature, sleep, and fatigue in response to secondary immune challenge, further demonstrating chronic immunological changes by viral infection. This is an important consideration in modeling, considering that while much of the population is exposed to EBV, there have been no studies to date of how latently infected individuals react to subsequent immune challenge. Understanding how EBV changes the immune system is important to understand how future illnesses and insults can affect individuals and applying proper treatments for these individuals after EBV infection to prevent chronic immune changes that can negatively affect responses to further insults. It is possible that global chronic immune changes could prime an individual to become chronically fatigued, however since all of our mice in these studies became fatigued, more studies are needed to explain the mechanisms of immune changes and what variables are important in the resulting changes due to viral infection.

Global implications and conclusions

Chronic fatigue is a disabling condition that is widespread and emotionally and physically devastating (Rakib et al., 2005). Due to the connection between EBV and CFS, and the high rates of exposure to EBV in the population, an alarming amount of the population is at risk for developing post-viral fatigue at some point in their lifetime.

Currently the mechanism of fatigue after a viral insult is largely unknown, however our studies show fatigue after a virus could be due to viral-induced changes in cytokines. Further studies are needed to understand the impact of the virus on resulting immune responses, and the impact of the virus on other systems involved in arousal, sleep, and thermoregulation, such as sympathetic and parasympathetic systems and the hypothalamic pituitary axis. It is also unclear what causes individuals to have a short post-viral fatigue state that lasts months and then slowly remits versus those who never have a remission of fatigue. Further longitudinal studies in humans are needed to explain this phenomenon.

Due to the many difficulties in studying EBV in humans as mentioned above, our studies have established MuGHV as a mouse model for studying post-viral fatigue in an objective and controlled manner. This model will allow us to address issues such as the impact of genetics, environment, social and physical stress, and immune status on the pathogenesis of fatigue after EBV. In addition, this model allows for further study of the mechanism for altered immune responses to subsequent immune challenge, and can be enhanced to include a broader range of pathogens. The results mentioned previously point more directly to substances that can inhibit somnogenic cytokine release centrally or peripherally, or block their effects. There are very few treatments for chronic fatigue, and they include mild exercise, behavioral therapies and anti-viral medications (Chambers, Bagnall, Hempel, & Forbes, 2006), as well as cytokine inhibitors (Kerr et al., 2007). This mouse model could be used to investigate treatments and prophylactics, including vaccines, cytokine inhibitors, viral inhibitors at specific time points during the viral infection, and behavioral tactics such as forced exercise, to decrease the fatigue and

other behavioral changes seen as a result of the virus. In addition, it is currently unknown how factors such as sleep, sleep quality, stress, and other environmental factors impact both the resulting behavior and immunology of the host in relationship to dose. This model would prove invaluable for studying these factors in a controlled environment.

The use of MuGHV can also enhance our understanding of how chronic fatigue can lead to depression. This model could be used to understand the emotional toll of chronic fatigue through simple behavioral and motivational tasks, therefore approaching better ways to prevent and treat depression in chronic fatigue patient populations. In addition, it could be used to test the intellectual toll on mice, as many chronic fatigue patients report "brain fog" and problems with learning (Vollmer-Conna et al., 1997). Overall this model creates a fantastic opportunity to research the devastating effects of post-viral fatigue, and restore quality of life to patients with CFS.

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APPENDIX

Statistical values for data presented in Chapters 3 and 4. Presented are degrees of freedom, F-values and resultant p values for those parameters that achieved statistical significance.

Table A1. Wheel running activity and temperature statistics during infection.

Variable	Dose	Day/Week	Comparison	Time Period (hrs)	df	F-value	p-Value
wheel	40,000 PFU	Week 1	HBSS	1_12	21	3.85	0.004
wheel	40,000 PFU	Week 2	HBSS	1_12	21	3.85	0.002
wheel	40,000 PFU	Week 3	HBSS	1_12	21	3.85	0.002
wheel	40,000 PFU	Week 4	HBSS	1_12	21	3.85	0.002
wheel	40,000 PFU	Week 1	HBSS	13_24	16	17.12	0.000
wheel	40,000 PFU	Week 2	HBSS	13_24	16	17.12	0.000
wheel	40,000 PFU	Week 3	HBSS	13_24	16	17.12	0.000
wheel	40,000 PFU	Week 4	HBSS	13_24	16	17.12	0.000
wheel	400 PFU	Week 3	HBSS	7_12	40	3.14	0.008
wheel	400 PFU	Week 4	HBSS	7_12	55	3.15	0.001
wheel	400 PFU	Week 1	HBSS	19_24	24	2	0.012
wheel	400 PFU	Week 2	HBSS	19_24	24	2	0.026
wheel	400 PFU	Week 3	HBSS	19_24	24	2	0.045
wheel	400 PFU	Week 4	HBSS	19_24	24	2	0.044
temp	40,000 PFU	Day 9	HBSS	7_12	18	8.7	0.004
temp	400 PFU	Week 3	HBSS	13_18	27	8	0.007
temp	400 PFU	Week 4	HBSS	13_18	27	8	0.000
temp	400 PFU	Week 2	HBSS	19_24	27	7.1	0.003
temp	400 PFU	Week 3	HBSS	19_24	27	7.1	0.004
temp	400 PFU	Week 4	HBSS	19_24	27	7.1	0.000
wheel	400 PFU	Week 3	HBSS	7_12	40	3.2	0.030
wheel	400 PFU	Week 4	HBSS	7_12	40	3.2	0.005

Table A2. Sleep variable statistics during infection.

Variable	Dose	Day	Comparison	Time Period (hrs)	df	F-value	p-Value
Nrem	40,000 PFU	Day 9	HBSS	1_6	28	12.9	0.000
Rem	40,000 PFU	Day 9	HBSS	1_6	28	5.1	0.014
Wake	40,000 PFU	Day 9	HBSS	1_6	28	12.5	0.010
Transitions	40,000 PFU	Day 9	HBSS	1_6	28	9	0.007
Nrem	40,000 PFU	Day 9	HBSS	7_12	26	14.4	0.000
rem	40,000 PFU	Day 9	HBSS	7_12	22	5.5	0.041
Wake	40,000 PFU	Day 9	HBSS	7_12	26	15.3	0.000
Transitions	40,000 PFU	Day 9	HBSS	7_12	25	12	0.014
delta power	40,000 PFU	Day 7	HBSS	13-16	50	1.34	0.029

Table A3. Food intake and Body weight statistics during infection.

Variable	Dose	Day/Week	Comparison	Time Period (hrs)	df	F-value	p-Value
weight	40,000 PFU	week 2	HBSS	n/a	8	17.1	0.002
food intake	40,000 PFU	day 8	HBSS	13-16	28	5.9	0.014
food intake	40,000 PFU	day 9	HBSS	13-16	28	5.9	0.033
food intake	40,000 PFU	day 10	HBSS	13-16	28	5.9	0.006
body weight	40,000 PFU	day 9	HBSS	13-16	12	5.98	0.038
body weight	40,000 PFU	day 10	HBSS	13-16	12	5.98	0.018
food intake	40,000 PFU	day 8	HBSS	13-16	28	5.9	0.014
food intake	40,000 PFU	day 9	HBSS	13-16	28	5.9	0.033
food intake	40,000 PFU	day 10	HBSS	13-16	28	5.9	0.006
body weight	40,000 PFU	day 9	HBSS	13-16	12	5.98	0.038
body weight	40,000 PFU	day 10	HBSS	13-16	12	5.98	0.018

Table A4. Cage/wheel activity, and temperature statistics after LPS injection.

Variable	Comparison	LPS DAY	Comparison	Hours	df	F value	P-value
cage	40,000 PFU	1	Saline	1_6	26	19.2	0.000
cage	40,000 PFU	1	Saline	7_12	18	15.6	0.000
cage	40,000 PFU	2	Saline	1_6	26	19.2	0.000
cage	40,000 PFU	2	Saline	7_12	18	15.6	0.015
wheel	40,000 PFU	1	Saline	1_6	9.2	20	0.000
wheel	40,000 PFU	2	Saline	1_6	9.2	20	0.024
cage	400 PFU	1	Saline	7_12	54	13	0.000
cage	400 PFU	2	Saline	13_18	54	10	0.038
temp	400 PFU	1	Saline	1_6	55	3.6	0.001
temp	400 PFU	1	Saline	7_12	38	3.6	0.003
wheel	400 PFU	1	Saline	1_6	70	11.3	0.000
wheel	400 PFU	1	Saline	7_12	63	7.9	0.000
wheel	400 PFU	2	Saline	1_6	70	11.3	0.000
wheel	400 PFU	2	Saline	7_12	63	7.9	0.004

Table A5. Within dose comparisons of sleep variables after Saline or LPS injection.

Variable	Comparison	LPS DAY	Comparison	Hours	df	F value	P-value
nrem	40,000 PFU	1	Saline	1_6	28	27.6	0.000
nrem	40,000 PFU	1	Saline	7_12	31	31.1	0.000
nrem	40,000 PFU	2	Saline	1_6	42	27.6	0.001
rem	40,000 PFU	1	Saline	1_6	42	6.95	0.001
rem	40,000 PFU	1	Saline	13_18	34	13.2	0.000
temp	40,000 PFU	1	Saline	1_6	7	3.6	0.024
temp	40,000 PFU	1	Saline	13_18	7	34.7	0.000
temp	40,000 PFU	1	Saline	19_24	9	7.2	0.000
temp	40,000 PFU	1	Saline	7_12	28	15.4	0.000
temp	40,000 PFU	2	Saline	1_6	7	3.6	0.015
temp	40,000 PFU	2	Saline	13_18	7	34.7	0.000
temp	40,000 PFU	2	Saline	19_24	9	7.2	0.000
temp	40,000 PFU	2	Saline	7_12	28	15.4	0.000
temp	40,000 PFU	3	Saline	13_18	7	34.7	0.000
temp	40,000 PFU	3	Saline	19_24	9	7.2	0.000
temp	40,000 PFU	3	Saline	7_12	28	15.4	0.000
temp	40,000 PFU	4	Saline	13_18	7	34.7	0.000
temp	40,000 PFU	4	Saline	19_24	9	7.2	0.000
temp	40,000 PFU	4	Saline	7_12	29	15.4	0.003
temp	40,000 PFU	5	Saline	13_18	7	34.7	0.000
temp	40,000 PFU	5	Saline	19_24	10	7.2	0.001
temp	40,000 PFU	5	Saline	7_12	30	15.4	0.015
transitions	40,000 PFU	1	Saline	1_6	37	35.4	0.000
transitions	40,000 PFU	1	Saline	7_12	38	15.4	0.000
transitions	40,000 PFU	1	Saline	13_18	27	11.8	0.000
transitions	40,000 PFU	1	Saline	19_24	16	13.1	0.000
wake	40,000 PFU	1	Saline	7_13	32	27.5	0.000
nrem	400 PFU	1	Saline	1_6	20	7.3	0.002
nrem	400 PFU	1	Saline	7_12	18	16	0.000
nrem	400 PFU	3	Saline	13_18	20	56.5	0.000

nrem	400 PFU	3	Saline	19_24	20	57	0.000
nrem	400 PFU	4	Saline	13_18	20	56.5	0.000
nrem	400 PFU	4	Saline	19_24	20	57	0.000
nrem	400 PFU	5	Saline	13_18	20	56.5	0.000
nrem	400 PFU	5	Saline	19_24	20	57	0.000
rem	400 PFU	3	Saline	13_18	8	9.2	0.003
rem	400 PFU	3	Saline	19_24	13	9	0.003
rem	400 PFU	4	Saline	13_18	8	9.2	0.048
rem	400 PFU	4	Saline	19_24	17	9	0.048
rem	400 PFU	5	Saline	13_18	8	9.2	0.008
rem	400 PFU	5	Saline	19_24	13	9	0.008
transitions	400 PFU	1	Saline	1_6	20	19	0.000
transitions	400 PFU	1	Saline	13_18	17	20	0.000
transitions	400 PFU	1	Saline	19_24	17	20	0.000
transitions	400 PFU	1	Saline	7_12	21	27.3	0.000
transitions	400 PFU	3	Saline	13_18	20	20	0.001
transitions	400 PFU	3	Saline	19_24	20	20	0.001
transitions	400 PFU	4	Saline	13_18	20	20	0.029
transitions	400 PFU	4	Saline	19_24	20	20	0.029
transitions	400 PFU	5	Saline	13_18	17	20	0.009
transitions	400 PFU	5	Saline	19_24	18	20	0.009
wake	400 PFU	1	Saline	1_6	21	7	0.003
wake	400 PFU	1	Saline	7_12	18	14.9	0.000
wake	400 PFU	3	Saline	13_18	20	58	0.000
wake	400 PFU	3	Saline	19_24	23	58	0.000
wake	400 PFU	4	Saline	13_18	20	58	0.000
wake	400 PFU	4	Saline	19_24	23	58	0.000
wake	400 PFU	5	Saline	13_18	20	58	0.000
wake	400 PFU	5	Saline	19_24	23	58	0.000

Table A6. Between dose comparisons of sleep variables after Saline or LPS injection.

Variable	Comparison	LPS DAY	Hours	df	F value	P-value
delta power	400 v 40,000 PFU	1	1_6	18	-5.6	0.000
delta power	400 v 40,000 PFU	1	13_18	12	-3.1	0.009
delta power	400 v 40,000 PFU	1	19_24	17	2.2	0.043
delta power	400 v 40,000 PFU	1	7_12	17	-2.4	0.027
rem	400 v 40,000 PFU	1	1_6	10	-2.4	0.035
trans	400 v 40,000 PFU	1	1_6	10	-3.1	0.012
trans	400 v 40,000 PFU	1	1_6	10	-3.2	0.009
delta power	Uninfected vs 40,000	1	1_6	11	-6	0.000
delta power	Uninfected vs 40,000	1	13_18	11	8.3	0.000
delta power	Uninfected vs 40,000	1	19_24	11	8.7	0.000
deltapwr	Uninfected vs 40,000 PFU	2	1_6	12	0.348	0.043
nrem	Uninfected vs 40,000	2	1_6	11	-2.5	0.029
rem	Uninfected vs 40,000	1	1_6	12	-2.5	0.028
rem	Uninfected vs 40,000	1	13_18	12	2.4	0.033
trans	Uninfected vs 40,000	1	1_6	12	-3.5	0.004
trans	Uninfected vs 40,000	1	7_12	12	-2.3	0.044
delta power	Uninfected vs 400	1	7_12	14	3.2	0.006
delta power	Uninfected vs 400	2	1_6	10	-3.1	0.044
trans	Uninfected vs 400	2	?	17	6.6	0.007

Table A7. Within and between dose comparisons of food and body weight

Variable	Comparison	LPS DAY	Comparison	df	F value	P-value
food intake	40,000 PFU	4	SALINE	14	-2.9	0.012
weight	40,000 PFU	1	SALINE	14	3.34	0.005
weight	40,000 PFU	2	SALINE	14	2.3	0.041
food intake	400 v 40,000 PFU	3	n/a	16	-2.3	0.034
weight	400 v 40,000 PFU	1	n/a	13	7	0.000
weight	400 v 40,000 PFU	3	n/a	8	-3.2	0.012